SEASONAL MITOCHONDRIAL METABOLISM AND ADENOSINE SIGNALING IN THE THIRTEEN-LINED GROUND SQUIRREL (*Ictidomys tridecemlineatus)*

A Thesis
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

Clair Elaine Hess

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Dr. Matthew T. Andrews

August 2017

Acknowledgements

I would like to thank and acknowledge my advisor, Dr. Matthew T. Andrews for without him, I would not be here today. You gave me a chance I never thought I would have and I cannot thank you enough. I would also like to acknowledge and thank my other committee members, Dr. Marshall Hampton and Dr. Jennifer O. Liang. You were always there for support and encouragement in addition to never making our meetings as intimidating as I thought they would be. Also, thank you to the Integrated Biosciences Graduate Program and the University of Minnesota Duluth Departments of Biology and Mathematics and Statistics. I have had amazing experiences as both a student and as a laboratory teaching assistant. I would also like to acknowledge the other members of the Andrews lab, especially: Mr. James Bjork, Mallory Ballinger, Kyle Anderson, Dr. Christine Schwartz, and Dr. Jessica Sieber. Your help, guidance, support, and friendship has made all the difference. Lastly, to my family, I cannot thank you enough for your endless love and support. The work was funded by grants to M.T. Andrews from the Department of Defense, National Institutes of Health, and the University of Minnesota McKnight Presidential Endowment.

Dedication

This thesis is dedicated to my family for always being there.

Abstract

The thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) undergoes extreme and rapid shifts in body temperature (T_b) during hibernation as it cycles between torpor (T_b ~ 5-10^{o}C) and interbout arousal (IBA, T_b ~37^{o}C). This requires regulated changes in the physiology of the tissues that produce much of the heat required during this shift, namely brown adipose tissue (BAT) and skeletal muscle. However, once normothermic body temperature is reached during arousal, production of additional heat is not needed; therefore, regulation of this whole-body heating and cooling is important. As hibernation involves coordinated responses from several organs, it is important to not only look at the function of each organ individually, but also how they influence the response of other tissues. To understand how the thirteen-lined ground squirrel regulates such extreme physiological shifts, I examined the influence of temperature and season on the function of thermogenic BAT at both the cellular and mitochondrial level by measuring respiration rates at several temperatures. Overall, I found isolated BAT mitochondria and adipocyte respiration is independent of season at each temperature measured. Since elevated adenosine has been suggested to serve as a potential means to inhibit BAT heat production by receptor-dependent processes, I also examined adenosine signaling by measuring the expression levels of proteins isolated from skeletal muscle involved in the production and transport of adenosine as well as the adenosine receptor in BAT. Two proteins involved in adenosine signaling which are differentially expressed (ADORA1 and AMPD1) could impact the function of BAT and other tissues.

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CHAPTER 1: Introduction

SECTION 1.1: HIBERNATION

Thermoregulation, or an organism's ability to maintain body temperature within a certain boundary, regardless of the surrounding temperature, is suggested to have been driven by selective pressures (Angilletta et al, 2010; Melvin and Andrews, 2009). The body temperature for mammals is typically between 35-38^{o}C. Endotherms, such as mammals, can use the heat produced from metabolism to maintain core body temperatures (Angilletta et al, 2010). The ability to maintain a relatively constant body temperature has given mammals the opportunity to remain active in a wider range of locations and temperatures, and become more highly organized physiologically (Kemp, 2005). However, this ability comes at an energetic cost.

Maintenance of regular body temperature can become too energetically costly for an organism when conditions become unfavorable, such as extreme environmental temperatures, low abundance of food, lack of water, etc. (Melvin and Andrews, 2009). Some small, endothermic mammals have adapted to survive these unfavorable conditions via various forms of heterothermy, such as daily torpor bouts or hibernation (Geiser and Körtner, 2010; Geiser, 1998; Geiser and Ruf, 1995). Over time, natural selection has favored animals capable of adapting in order to survive challenging and varying conditions (Melvin and Andrews, 2009). However, with mammalian hibernation also being discovered in warmer and tropical climates, in addition to cold climates, the idea that energy conservation being the main reason for hibernation is being challenged (Geiser and Körtner, 2010; Barnes, 1989; Geiser and Stawski, 2011). Additionally, some studies have revealed that hibernation can occur during the summer or in times when no

obvious stress is observed, such as lack of food or low energy (Geiser and Körtner, 2010; Geiser and Brigham, 2012). Thus, the need to conserve energy may not be the only purpose of hibernation, despite the observation that many heterotherms use torpor during energetically stressful times and/or when food is lacking (Geiser and Brigham, 2012). During such times, body temperatures and metabolic rates are lowered to conserve energy for survival and, consequently, they enter torpor (Geiser and Ruf, 1995).

Torpor has evolved as an adaptive response to irregular environmental conditions and lack of resources (Melvin and Andrews, 2009). Despite the ancient evolutionary split between the three Mammalian subclasses, 180 million years ago in Prototheria and Metatheria and 120 million years ago between Metatheria and Eutheria, torpor is found in all three, but is limited to more ancestral groups (Geiser, 1998). There are three types of torpor patterns recognized in these species; 1) daily torpor, which can last for several hours with a minimum body temperature average around 17°C, although this is variable depending on the species (Geiser and Körtner, 2010; Geiser, 1998), 2) another less common type of torpor pattern, usually described as hibernation instead of torpor, occurs in which body temperature usually ranges between 28-30°C while still undergoing dramatic reductions in metabolism, as demonstrated by larger heterothermic endotherms such as bears (Geiser, 1998; Ruf and Geiser, 2015; Tøien et al, 2011). The third, and perhaps most well-known, pattern of torpor is deep and extended torpor or hibernation; which will be the focus of this thesis.

Hibernation is a method of conserving energy in which controlled decreases in motor activity, body temperature, basal metabolic rate (BMR), renal function, oxygen

consumption, carbon dioxide production, and heart rate occur for a few days up to five weeks, depending on the species (Geiser and Körtner, 2010; Geiser and Ruf, 1995; Carey et al, 2003; Geiser, 2004). During torpor, metabolic rates decrease to 2-4% of normal rates, heart rate decreases to 2-3% of normal, and respiration rates decrease in a similar pattern, all while body temperature remains a few degrees above ambient temperature (Table 1.1; Carey et al, 2003). Hibernation typically occurs seasonally for five to seven months, from late summer/fall to late winter/spring, but is interrupted by occasional periods of rewarming, termed interbout arousal (IBA), that usually last less than one day (Geiser, 2004). These periodic arousals are energetically costly; therefore, to have enough energy to survive the hibernation season, hibernators rely on either cached food or body fat stores (Carey et al, 2003). Hibernators, such as chipmunks, store food that is consumed upon arousal (Carey et al, 2003). The thirteen-lined ground squirrel, *Ictidomys tridecemlineatus*, and other hibernating animals that rely on fat stores do not consume any food during the hibernation months (Carey et al, 2003). Instead their metabolism is modified to rely on fatty acids and glycerol from lipolysis of triglycerides that have accumulated in white adipose tissue (WAT) prior to the hibernation season (reviewed in: Melvin and Andrews, 2009; Geiser and Körtner, 2010; Geiser, 2004; Tashima et al, 1970). Although the animal's body weight nearly doubles as WAT accumulates in preparation for hibernation, the fat stores would not be enough to maintain a state of homeothermy throughout the circannual cycle (Carey et al, 2003).

Even when energetically costly arousals are considered, *Geiser* (2004) argues that energy expenditure in mammals is still reduced to lower than 15% of what would have

been spent throughout the hibernation season if torpor were not utilized. This adaptation allowing for a reduction in energy usage permits hibernators to survive extended periods of unfavorable conditions, such as low food availability and extremes in temperature (Geiser and Körtner, 2010; Geiser, 2004; Bieber et al, 2013).

Model Organism: Thirteen-lined Ground Squirrel

The thirteen-lined ground squirrel is a small, obligate hibernator that is distributed across North America in the grasslands and prairies, extending from central Canada to the Gulf of Mexico. All animals used in these studies were either captured in central Minnesota or born in the lab to mothers captured in the same location. In this northern habitat, winters are long and cold with limited food resources, therefore, the circannual cycle of these squirrels is portioned into two phases: homeothermy and heterothermy (Otis et al, 2011; Figure 1.1). During homoeothermic periods (late spring to late fall), squirrels are active and have constant, normothermic body temperature and physiological rates (Figure 1.1). Emergence from hibernation in the spring is thought to be triggered by activity changes in the reproductive system as squirrels reproduce once a year in early spring, shortly after hibernation (Carey et al, 2003; Hut et al, 2014). After reproduction, squirrels spend the rest of summer and fall preparing for hibernation by increasing stores of WAT (Carey et al, 2003). Throughout this phase of hyperphagia, the squirrels' body weight doubles or even triples (Carey et al, 2003). This period of hyperphagia is followed by hypophagia in mid to late fall when squirrels are still active, but decrease their food consumption (Schwartz et al, 2015a). This is accompanied by shallow torpor

bouts where squirrels drop their body temperature to ambient temperature for less than a day (Schwartz et al, 2013; Figure 1.1).

Entrance into torpor and a gradual decrease in body temperature are initiated by active, coordinated events in which metabolism is suppressed and the hypothalamic set point is lowered, therefore reducing metabolic thermogenesis and sympathetic activation of thermogenic brown adipose tissue (BAT; Heldmaier and Ruf, 1992; Snapp and Heller, 1981). Throughout the hibernation season a constant cycle between torpor and IBA occurs (Figure 1.1). Torpor lasts anywhere from 7-14 days, on average, and is characterized by body temperatures around 2-10^{o}C, heart rate falling to 2-10 beats per minute, and oxygen consumption 2-3% that of normal, euthermic levels (Andrews, 2007; Figure 1.1). The extreme decreases in body temperature and other physiological processes that hibernators endure are highly stressful or even deadly for animals that cannot hibernate. In torpor, there is almost no physiological process that functions the same as it does at normothermic values (Carey et al, 2003). However, several necessary functions continue at suppressed rates even at body temperature values near 0^{o}C including the central nervous system, cardiac function, and lipolysis in adipose tissues, among others (Carey et al, 2003). Processes that must function for survival have mechanisms in place that allow activity; however, these mechanisms are currently unknown.

In the thirteen-lined ground squirrel, torpor is periodically interrupted by IBAs, in which whole body rewarming facilitates the return to normothermic rates with a heart rate around 300-400 beats per minute, oxygen consumption returns to normal, and body

temperature increases to approximately 37^{o}C in about 2-3 hours (Carey et al, 2003; Hampton et al, 2010; Figure 1.1; Table 1.1). During arousal from torpor to IBA, the animal rewarms to normothermic temperatures in approximately 2.8 hours (Hampton et al, 2010; Schwartz et al, 2015a; Figure 1.4A). This energetically costly arousal is maintained for 12-24 hours, after which the animal returns to torpor (Carey et al, 2003). Currently, the reason(s) for IBAs are unknown. The longest torpor periods occur between January and February in thirteen-lined ground squirrels (Schwartz and Andrews, 2013; Figure 1.1). As spring approaches, the squirrels' WAT mass is greatly depleted (Table 1.1). As they return to a homoeothermic state they have much leaner bodies, with body temperature, heart rate, oxygen consumption, and metabolism resuming normal levels (Table 1.1; Carey et al, 2003).

Functional Variations

In hibernators, several biochemical processes rapidly resume normal activity upon rewarming and exploit lower temperatures to decrease reaction rates (Carey et al, 2003). Principally, changes in activity states are achieved by covalently modifying proteins, typically through reversible phosphorylation (Storey and Storey, 1990). These modifications result in changes in the regulation of enzymes and activity state of functional proteins through molecular signals (Storey and Storey, 1990). Additional enzyme activity modification can be achieved via decreases in levels of substrates (Storey and Storey, 1990). Some of the processes that shift in activity include transcription and translation, mitochondrial respiration, and immune response, among others (Carey et al,

2003; van Bruekelen and Martin, 2001). Other processes that rely on enzyme function, like the citric acid cycle or glycolysis, also have slow reaction rates due to temperature and potential post-translational modifications (Nicholls and Locke, 1984; Staples, 2014; Carey et al, 2003; Mathers et al, 2016; Hofer and Wenz, 2014). A change in fuel utilization occurs from carbohydrates to fatty acids because of these decreases (Nicholls and Locke, 1984; Staples, 2014; Carey et al, 2003).

In the summer months, when food is abundant, the primary fuel is carbohydrates (Carey et al, 2003). Glucose is either used for energy through ATP production or stored as glycogen to be consumed later (Carey et al, 2003). During hibernation when food is scarce, but fat stores are abundant, squirrels shift fuel utilization pathways to rely principally on lipids instead of glucose (Storey and Storey, 2004). This shift is evidenced by the increasing fat stores pre-hibernation followed by lack of feeding throughout hibernation and reduction of WAT mass, also evidenced by much leaner appearance upon emergence in the spring (Table 1.1; Andrews, 2007).

Fatty acids are liberated from WAT before being metabolized via β-oxidation (Carey et al, 2003). An important enzyme responsible for lipolysis, pancreatic triacylglycerol lipase, has higher activity levels at low temperatures (Bauer et al, 2001) and has significantly higher mRNA levels in BAT during April than the rest of the year (Hampton et al, 2013). Pyruvate dehydrogenase kinase isoenzyme 4 and pancreatic triacylglycerol lipase function together to facilitate the switch in fuel from carbohydrates to fatty acids, respectively (Carey et al, 2003). *Pyruvate dehydrogenase kinase isoenzyme 4* mRNA is differentially expressed in both BAT and WAT with significantly

higher levels in April compared to October (Hampton et al, 2013). Once the fatty acids are converted to Acetyl-CoA, the citric acid cycle uses Acetyl-CoA to produce the fuels required by the electron transport system (ETS) in mitochondria. The mitochondrial enzymes of the citric acid cycle provide the ETS with the fuels required to produce ATP or in BAT, heat.

Section 1.1: Figures and Tables

Figure 1.1: Thirteen-lined ground squirrel body temperature throughout circannual cycle. Body temperature (solid line, measured by surgically implanted transmitter) compared to ambient temperature (dashed line) across the circannual cycle with corresponding time points. Abbreviations: IBA; interbout arousal. (Adapted and modified from Hampton et al, 2013)

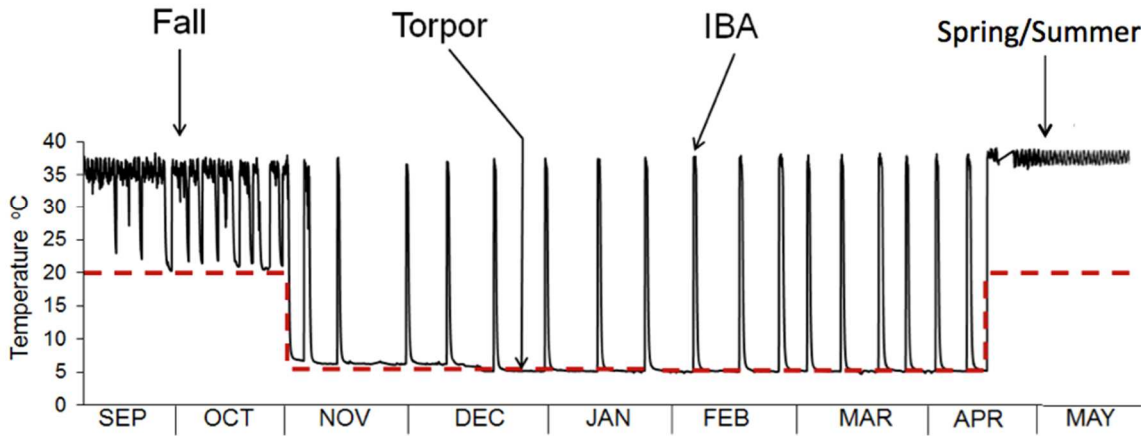


Table 1.1: Physiological characteristics of experimental collection time points. Description of physiological characteristics in thirteen-lined ground squirrels at each time point. Abbreviations: IBA; interbout arousal, Tb; body temperature, VO_2; maximal oxygen consumption, HR; heart rate, bmp; beats per minute. (Adapted and modified from Schwartz et al, 2012)

Collection Point		Characteristics
Fall	Hyperphagic	Increased food consumption and white adipose stores, Tb: 37°C, VO_2: 100%, HR: 300-400 bpm
	Hypophagic	Little to no food consumption, maximum white adipose stores, starting shallow torpor bouts, Tb: 37°C to ambient temperature, heart rate and metabolism slow
Hibernation	Torpor	Tb: A few degrees above ambient, VO_2: 2-3% normothermic rate, HR: 3-10 bpm, MR: 2-4% normal rate
	IBA	No food consumption, Tb: 37°C, VO_2: 100%, HR: 300-400 bpm
Spring/ Summer active	Spring	Increasing food consumption, low white adipose stores, reproductively active, Tb: 37°C, VO_2: 100%, HR: 300-400 bpm
	Summer	Increasing food consumption, Tb: 37°C, VO_2: 100%, HR: 300-400 bpm

SECTION 1.2: MITOCHONDRIA

The content in this section (1.2) is largely derived from Nicholls and Ferguson, 2013.

Fats, carbohydrates, and protein can be used as fuel for cells to make chemical energy in the form of ATP via oxidative phosphorylation in mitochondria (Figure 1.2). Although some ATP is produced by substrate level phosphorylation, the large majority of ATP is produced via ATP-synthase coupled to a series of oxidation-reduction reactions catalyzed by membrane-bound enzymes as part of the ETS (Figure 1.3). The process of ATP production by mitochondria was proposed by Peter Mitchell in 1961 and is referred to as the chemiosmotic theory. Mitochondria have two membranes; an outer membrane with pores that are non-specific and allow solutes with molecular weights under 10 kDa to pass through. The space separating the outer and inner membranes, the intermembrane space, contains a high proton concentration due to passing of electrons through inner membrane-bound ETS protein complexes. The inner membrane is the energy-transducing membrane and supports the proteins of the ETS. The mitochondrial matrix, encircled by the inner membrane, contains enzymes for β-oxidation and the citric acid cycle, except succinate dehydrogenase (Complex II), which is bound to the inner membrane. Other proteins that aid in the transport of fatty acids and pyruvate also span the mitochondrial membranes.

Electron transport in respiring mitochondria results in passing electrons through a series of oxidation-reduction reactions. These electrons are derived from products of the citric acid cycle, β-oxidation, and other pathways. Electrons are donated by mobile electron carriers, nicotinaminde adenine dinucleotide (NADH, reduced form) and flavin

adenine dinucleotide ($FADH_2$, reduced form), which gain electrons from the citric acid cycle and β-oxidation. The carriers then donate the electrons to protein complexes in the ETS resulting in the reduction of a final electron acceptor, oxygen (O_2), which is reduced to water.

Electron Transport

Electron transport is catalyzed by a series of protein complexes bound to the inner mitochondrial membrane (Figure 1.3). The four main complexes (I-IV) and ATP-synthase (Complex V) make up the ETS. Oxidative phosphorylation begins with the passing of electrons from NADH to NADH dehydrogenase (Complex I) to regenerate NAD^+. Complex I then passes electrons to fat-soluble ubiquinone and results in four protons being pumped to the inter membrane space. As succinate is converted to fumarate in the citric acid cycle, $FADH_2$ is produced and electrons are passed to succinate dehydrogenase (Complex II). These electrons are also passed to ubiquinone, but Complex II does not contribute to the proton gradient built up in the intermembrane space. Complex II is also the only component of the citric acid cycle and is membrane bound. Ubiquinone then transfers electrons to cytochrome bc1 complex/cytochrome c oxidoreductase (Complex III), which also pumps four protons into the intermembrane space. Electrons are passed from Complex III to water-soluble cytochrome c. Cytochrome c then passes electrons to cytochrome c oxidase (Complex IV). During this process, two protons are pumped to the intermembrane space and oxygen is reduced to water. The high proton gradient that forms in the intermembrane space due to electron

transfer is dissipated as protons flow down their concentration gradient through ATP synthase (Complex V) producing ATP in the process. The proton circuit is controlled by proton flow back into the mitochondrial matrix principally through Complex V.

This is coupled respiration since ATP synthesis is coupled to the ETS activity. While some ATP is produced in pathways besides the mitochondrial ETS, most of the ATP in the body is produced in mitochondria by ATP-synthase when coupled to a series of oxidation-reduction reactions catalyzed by membrane-bound enzymes. However, low temperatures reached in torpor can impact the function of some citric acid cycle enzymes (Carey et al, 2003; Nicholls and Locke, 1984; Staples, 2014) and not all mitochondria function to yield primarily ATP.

Bioenergetics measurements

The function of the ETS can be investigated by measuring the rate of oxygen consumption as electrons are passed through the system ending with the reduction of oxygen to water. Since each electron transport protein is more electronegative than the previous, the rate of electron transfer naturally increases whenever there is ample substrate, oxygen, and ADP. Oxygen consumption is most commonly measured with an oxygen electrode, first applied to mitochondrial experiments by Chance and Williams in 1955. Oxygen consumption measurement rates can be described in terms of states defined by *Chance and Williams* (1955), which follow the addition of reagents throughout an experiment in order.

State 1 corresponds to the basal rate of mitochondrial respiration when isolated mitochondria are alone in the reaction chamber. Although some ADP and fuel may be present in the mitochondrial matrix after mitochondrial isolation, these concentrations are usually low and rapidly used until equilibrium is reached when mitochondria are placed in the chamber with respiration buffer. Upon addition of ADP, but no exogenous substrate for fuel, *state 2* respiration is achieved. *State* 3 respiration rates occur when both ADP and substrate are present in coupled mitochondria resulting in typically rapid respiration and ATP synthesis rates. Usually the substrate is added before ADP and in excess with saturating oxygen levels. When all ADP has been converted to ATP, *state 4* respiration rate is obtained. *State 4* respiration is also known as non-phosphorylating respiration rate because ATP synthesis has no influence on the rate. This rapid rate can also be achieved with the addition of a chemical uncoupler before all ADP is converted to ATP. Non-phosphorylating respiration can also be achieved naturally in mitochondria with an uncoupling protein. Presence of an uncoupler, protein or chemical, results in uncontrolled respiration as the proton gradient is dissipated through an ionophore or by blocking ATP synthase. Uncoupled respiration rates are the maximal rates that can be achieved in isolated mitochondria. In mitochondrial respiration experiments, typically only *states 3* and *4* are used. *State 5* is reached when there is no more oxygen present.

Section 1.2: Figures and Tables

Figure 1.2: Metabolic pathways of mitochondria providing fuels for the electron transport system. Glycolytic product pyruvate and fatty acids are transported into the mitochondrial matrix from the cytoplasm (transporters not shown). 1) Pyruvate undergoes oxidation to produce Acetyl CoA and NADH. 2) Acetyl CoA, from the previous step, and from β-oxidation, enters the citric acid cycle. 3) Acetyl CoA undergoes a series of chemical, catabolic reactions facilitated by various enzymes that results in the production of electron carriers, NADH and $FADH_2$. 4) NADH and $FADH_2$ from pyruvate oxidation, β-oxidation, and the citric acid cycle pass electrons to Complexes I and II of the electron transport system for oxidative phosphorylation. The electron transport system produces ATP, heat, and water.

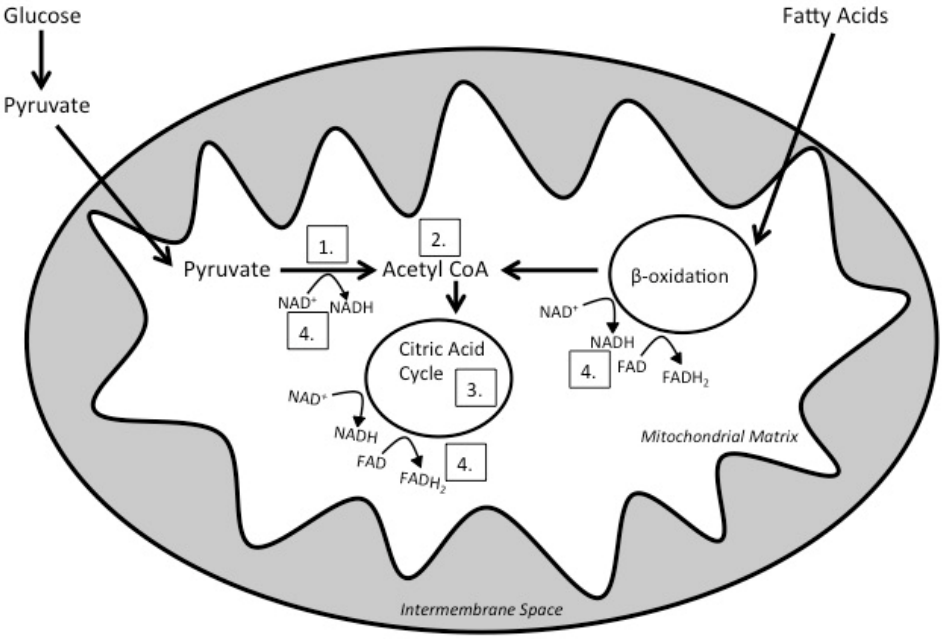
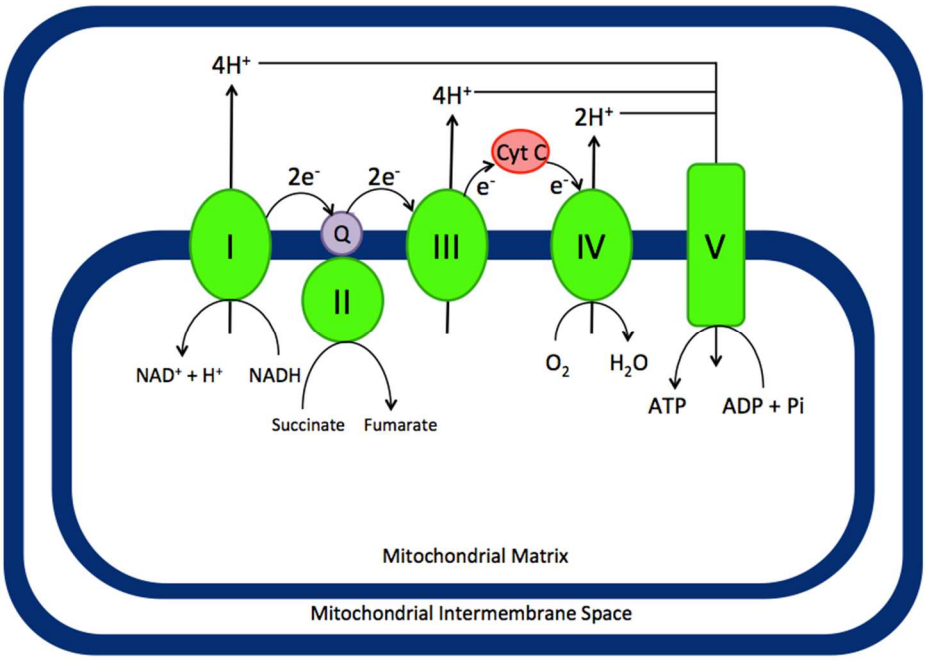


Figure 1.3: Isolated mitochondrial electron transport system. Mitochondrial electron transport system proteins are located on the inner mitochondrial membrane and facilitate the transfer of electrons from the oxidation of energetic substrates. Abbreviations: I; Complex I/NADH dehydrogenase, II; Complex II/Succinate Dehydrogenase, III; Complex III/Cytochrome bc1 complex, IV; Complex IV/Cytochrome oxidase, V; Complex V/ATP synthase, Q; ubiquinone, Cyt C; cytochrome C, e^-; electron, H^+; proton.



SECTION 1.3: BROWN ADIPOSE TISSUE

Brown adipose tissue, found in hibernating mammals and other non-hibernators, primarily functions as a thermogenic tissue (Cannon and Nedergaard, 2004). The presence of fat droplets aids in BAT's ability to convert chemical energy to heat via non-shivering thermogenesis (Cannon and Nedergaard, 2004). Brown adipose tissue contains numerous small lipid droplets and a high density of capillaries to supply the abundant mitochondria with reactants and delivery of product (heat; Cannon and Nedergaard, 2004). In comparison, the other type of fat found in mammals, WAT, contains many large lipid droplets, but few mitochondria since its main function is fat storage (Cannon and Nedergaard, 2004). Brown adipose tissue only makes up to 5% of the body mass in small rodents, such as ground squirrels, yet it can increase the entire body's respiration up to 10-fold due to BAT respiration (Nicholls and Ferguson, 2013). To fuel BAT thermogenesis, the abundance of triglyceride stores that accumulated in WAT prior to the hibernation season are used (Cannon and Nedergaard, 2004; Carey et al, 2003; Melvin and Andrews, 2009; Geiser and Körtner, 2010; Geiser, 2004; Tashima et al, 1970). Mitochondrial enzymes from BAT have a greater ability to oxidize fatty acids to acetyl groups to be used as fuel during hibernation (Bernson, 1976). However, the weight of BAT varies seasonally (Ballinger et al, 2016). Even though ground squirrel BAT is not particularly abundant, the ability to break down fat and produce heat via non-shivering thermogenesis proves to be advantageous for several reasons.

In mammals, BAT produces heat that warms surrounding blood, which is pumped throughout the body warming other tissues (Cannon and Nedergaard, 2004). Brown

adipose tissue is most active during arousal from torpor to IBA when the animal rewarms to normothermic temperatures in approximately 2.8 hours (Hampton et al, 2010; Schwartz et al, 2015a; Figure 1.4A). This rapid rewarming mediated by BAT is facilitated by an increasing heart rate, as warm blood circulates the body increasing body temperature 20^{o}C in less than an hour (Schwartz et al, 2015b; Figure 1.4B). Brown fat adipocytes do not function in isolation; BAT is controlled by nerves, chemical signals, and depends on lipids and oxygen as substrates (Cannon and Nedergaard, 2004). Norepinephrine and adenosine are thought to control BAT function with opposing roles. Norepinephrine initiates the signaling cascade that facilitates brown adipocyte function as a heat generator (Cannon and Nedergaard, 2004). Norepinephrine is released from the sympathetic nervous system and upon binding β_3-adrenergic receptors coupled to G-proteins on the brown adipocyte plasma membrane mediates thermogenic activity.

Norepinephrine binding to β_3-adrenergic receptors triggers adenylate cyclase activity, which catalyzes ATP to cyclic AMP (cAMP; Cannon and Nedergaard, 2004). Through a signaling cascade, perlipin (a protective protein coding on adipocyte lipid droplets) is inactivated by phosphorylation (Cannon and Nedergaard, 2004). When perilipin is phosphorylated, it dissociates from triglyceride droplets, so they are no longer protected, and therefore, exposed to hormone sensitive lipase, which breaks triglycerides into glycerol and free fatty acids stored within brown adipocytes (Cannon and Nedergaard, 2004). These fatty acids are transported across the mitochondrial membranes and as the concentration of fatty acids increase, they bind to and activate mitochondrial uncoupling protein 1 (UCP1); a six-transmembrane protein located on the

inner mitochondrial membrane (Cannon and Nedergaard, 2004; Fedorenko, 2012; Nedergaard et al, 2001).

Fatty acid binding to UCP1 causes activation, and a conformational shift, allowing UCP1 to be proton conducting which results in heat generation (Cannon and Nedergaard, 2004; Nicholls and Ferguson, 2013). When the norepinephrine signal is low, lipolysis has stopped, and the pool of free fatty acids has been oxidized, BAT respiration decreases (Cannon and Nedergaard, 2004; Nicholls and Ferguson, 2013). Additionally, when purine di- and triphosphate nucleosides bind to UCP1, function is inhibited (Nicholls and Ferguson, 2013).

With UCP1 present, the mitochondrial ETS is uncoupled from ATP synthesis and the proton gradient is rapidly dissipated via UCP1. This allows energy to be released as heat rather than being converted into ATP resulting in increased respiration rates (Nicholls and Ferguson, 2013; Staples, 2014; Figure 1.5). In tissues that are not considered thermogenic and UCP1 is not present, a small amount of heat will be produced as a byproduct of respiration, but ATP is the primary product in non-thermogenic tissues. The role of UCP1 was supported when studies showed that in the absence of UCP1, thermogenesis could not be signaled by norepinephrine (Matthias et al, 2000). Furthermore, UCP1 is only expressed in BAT (Hampton et al, 2013). In the thirteen-lined ground squirrel, BAT mitochondria differentially express UCP1 between hibernation (includes both torpor and IBA) and April (Hampton et al, 2013).

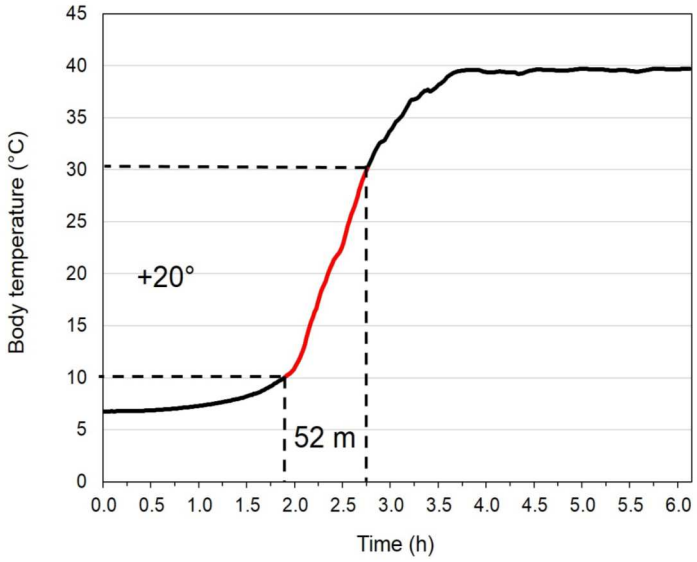
BAT may produce more heat with more mitochondria present, since the cells have a higher respiratory capacity (Kaaman et al, 2007). On the other hand, there could be

some regulatory mechanism(s) in place that allows the tissue to function the same regardless of the concentration of mitochondria. One approach to quantifying mitochondria is by measuring mitochondrial DNA copy number. When comparing the relative abundance of DNA sequences unique to the mitochondrial genome to the DNA found in the nuclear genome of thirteen-lined ground squirrels, results show there is a higher number of mitochondrial sequences in both fall active (September-October) and hibernation (torpor and IBA) compared to spring (April-May; Ballinger et al, 2016). These results suggest there are more BAT mitochondria present during fall and hibernation, which could help explain why BAT is so efficient at generating heat during colder months.

Section 1.3: Figures and Tables

Figure 1.4: Arousal cycle from torpor to interbout arousal (IBA). Body temperature and heart rate during arousal measured by a surgically implanted transmitter. A) Body temperature increase compared to time upon arousal from torpor to IBA in thirteen-lined ground squirrels. Body temperature can increase 20ºC in as little as 52 minutes as concurrent increases in heart rate (B, red line) facilitate rapid body temperature increases (B, black line). (Panel (A) is modified from Schwartz et al, 2015a; Panel (B) is modified from Schwartz et al, 2015b)

A.



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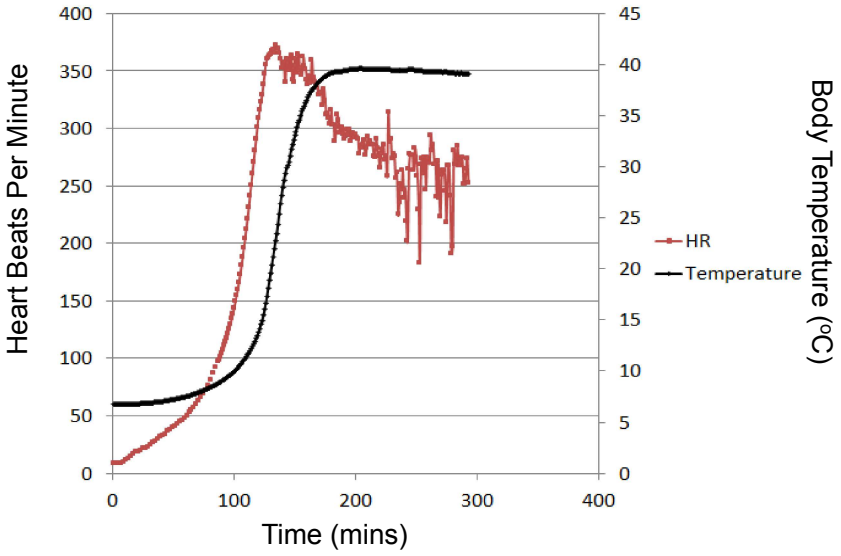
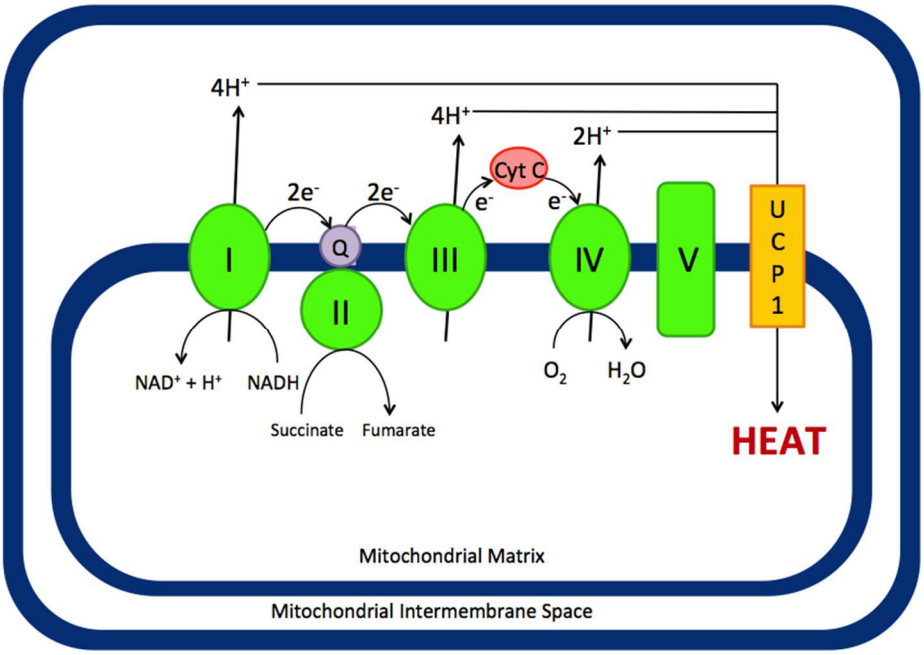


Figure 1.5: Brown adipose tissue mitochondrial electron transport system. Mitochondrial electron transport system proteins are located on the inner mitochondrial membrane and facilitate the transfer of electrons from the oxidation of energetic substrates. Abbreviations: I; Complex I/NADH dehydrogenase, II; Complex II/Succinate Dehydrogenase, III; Complex III/Cytochrome bc1 complex, IV; Complex IV/Cytochrome oxidase, V; Complex V/ATP synthase, Q; ubiquinone, Cyt C; cytochrome C, e^-; electron, H^+; proton, UCP1; uncoupling protein 1.



SECTION 1.4: ADENOSINE

As hibernation is a whole-body phenomenon, other tissues besides BAT play an important part in the hibernation phenotype. Another essential tissue is skeletal muscle. Found body wide, skeletal muscle is not only tested by, but also contributes to the animal being able to achieve the extreme physiological changes of hibernation. One way in which skeletal muscle contributes to the animal's survival during hibernation is through shivering thermogenesis as the animal rewarms from torpor to IBA. When shivering thermogenesis occurs, a large amount of ATP is used and thus, AMP accumulates. If levels of AMP are high, the ratio of AMP: ATP increases thereby stimulating phosphorylation and activation of AMP-Activated Protein Kinase (AMPK; Figure 1.6; Hardie et al, 2012). This activation results in targeting downstream processes aimed at increasing ATP production while decreasing ATP utilization (Hardie et al, 2012) which is especially important throughout hibernation when energy conservation is crucial.

Previous studies investigating the role of AMP and torpor induction in mice found that when administered, a hypothermic or "torpor-like" state resulted, characterized by reduced body temperature (Swoap et al, 2007; Zhang et al, 2006). When mice were injected with other adenine nucleotides (ATP and ADP), adenosine, and inosine nucleotide IMP, a similar hypothermic response occurred, but the response was more pronounced with adenosine and adenine nucleotides compared to IMP (Swoap et al, 2007). If the adenosine receptor was blocked with aminophylline (non-selective adenosine receptor antagonist) prior to administration of AMP or adenosine, no hypothermic response occurred (Swoap et al, 2007).

AMP levels can be lowered through the action of AMP Deaminase. The skeletal muscle specific isoform of AMP Deaminase, AMPD1, catalyzes the rate limiting step in the metabolism of AMP to IMP and releases ammonia as a byproduct (Figure 1.6; Plaideau et al, 2014). AMPD1 is differentially expressed between April active animals compared to fall and hibernation (Anderson et al, 2016). Thus, during hibernation, with low levels of AMPD1, accumulating AMP is converted to adenosine (Figure 1.6; Zhang et al, 2006; Plaideau et al, 2014). *Swoap et al* (2007) suggest it is adenosine, coupled with activation of the adenosine receptor, and not adenine nucleotides (AMP, ADP, ATP) that induces the hypothermic response since the adenine nucleotides are de-phosphorylated by 5'-Nucleotidases (NT5s) to adenosine (Figure 1.6). We found *NT5* mRNA expression is high in skeletal muscle, although not differently expressed, so there is an increased potential for more adenosine production.

Adenosine is known to be important in homeostasis as a regulator through receptor-dependent processes such as sleep, hibernation, and thermoregulation (Drew and Jinka, 2013; Swoap et al, 2007; Zhang et al, 2006). Adenosine can affect components of the cardiovascular system, including lowered heart rate; this could reduce the levels of circulating glucose resulting in lower metabolic rate and thus, decreased heat production and body temperature (Swoap et al, 2007). Throughout hibernation, entrance into torpor is facilitated by a reduction in the thermoregulatory set point, which causes a shift in the thermoneutral zone resulting in lower body temperature (Snapp and Heller, 1981). To lower body temperature, heat producing processes that help to maintain higher body temperatures must be inhibited, such as shivering and non-shivering thermogenesis.

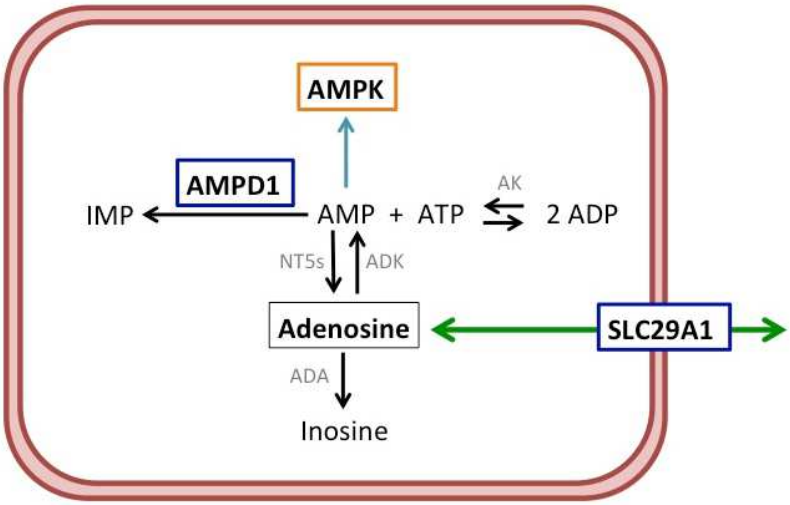
Brown adipose tissue non-shivering thermogenesis can be inhibited by adenosine through interaction with Adenosine A_1 Receptor (ADORA1; Figure 1.6; Hampton et al, 2013), which constricts sympathetic activation. In BAT, *ADORA1* mRNA shows highest expression in torpor and lowest in April (Hampton et al, 2013). Once adenosine binds to ADORA1 on the cell membrane of brown adipocytes, adenylate cyclase activity is inhibited (Cannon and Nedergaard, 2004; Hampton et al, 2013). This inhibition prevents the production of cyclic AMP (cAMP) and therefore, the function of downstream processes, ending with repression of non-shivering thermogenesis mediated by BAT mitochondria (Cannon and Nedergaard, 2004; Hampton et al, 2013).

Skeletal muscle intracellular adenosine could accumulate when there is a reduced ability to metabolize increasing AMP levels, for instance during shivering thermogenesis when ATP utilization is high. This could occur during hibernation when AMPD1 levels are low and therefore, AMP is not deaminated to IMP. A potential mechanism for the release of excess adenosine from skeletal muscle and into circulation is through the Equilibrative Nucleoside Transporter 1/Solute Carrier Family 29, Member 1 SLC29A1 (Figure 1.6; Beal et al, 2004). *SLC29A1* mRNA is differentially expressed in BAT and skeletal muscle with the levels being highest in hibernation (torpor and IBA; Hampton et al, 2013; Vermillion et al, 2015) further supporting the importance of adenosine handling throughout the circannual cycle and, especially, hibernation. Adenosine is transported down its concentration gradient via SLC29A1 in either direction; when adenosine levels are high intracellularly, it is released into circulation (Figure 1.6; Drew et al, 2016). The

opposite is also thought to occur; when extracellular levels of adenosine accumulate, it is transported into cells (Figure 1.6; Drew et al, 2016).

Section 1.4: Figures and Tables

Figure 1.6: Metabolic pathway in skeletal muscle involved in mediating intracellular adenosine levels. Adenosine is transported down its concentration gradient via Equilibrative Nucleoside Transporter/Solute Carrier Family 29, Member 1 (SLC29A1) in either direction. Intracellularly, adenosine can be deaminated to inosine by Adenosine Deaminase (ADA) or phosphorylated to AMP, with an input of energy (ATP) by Adenosine Kinase (ADK). AMP can be dephosphorylated to adenosine by 5'-Nucleotidases (NT5s). Adenylate Kinase (AK) catalyzes the conversion of AMP and ATP to two ADP molecules. AMP Deaminase 1 (AMPD1) could also metabolize AMP to IMP. A high AMP: ATP triggers activation, via phosphorylation, of AMP-Activated Protein Kinase (AMPK), resulting in targeting downstream processes aimed at increasing ATP production while decreasing ATP utilization.



SECTION 1.5: RESEARCH QUESTIONS

During seasons of activity, mammalian hibernators' physiological processes do not differ greatly from that of other mammals. On the other hand, when comparing circannual cycles, hibernators can significantly alter and maintain physiological states that are not possible in non-hibernators. We can begin to understand how ground squirrels can survive such extreme physiological shifts by examining how temperature is maintained during the suppressed state and how each season leads to changes in expression levels and cellular function.

Brown adipose tissue has a thermogenic role and functions across all ranges of temperature; from near freezing in torpor to normothermic levels. However, how respiration rates and overall function within brown adipocytes and mitochondria are impacted by temperature is not understood. The thermogenic capacity of BAT could be better understood by examining both individual cellular components (i.e. mitochondria) and how the cell functions as a whole (adipocyte) to the overall respiratory capacity and heat production of the tissue.

However, heat production via non-shivering thermogenesis is not always needed, for example when the squirrel is in torpor or at normothermic body temperatures, therefore, being able to mediate this process is crucial. A deeper understanding of how non-shivering thermogenesis is regulated can be gained by examining some of the enzymes that control hormone signaling, and how season changes enzyme expression levels. Furthermore, by looking at the contribution of other tissues and the influence that

multiple tissues and their products have, provides further insight on whole organismal metabolism.

A new approach to understanding BAT function is introduced by conducting experiments that account for the range of temperature that squirrels experience across the circannual cycle. Integrating thermodynamics and energy flow into the study of respiration rates when considering season, tissue, and chemical availability could improve the understanding of how hibernators adjust to such extreme physiological changes without ill effect or injury. Considering how well hibernators can use body temperature to increase survival, understanding these mechanisms could be applicable to human conditions as well. One application of this improved understanding of the body's response to cold temperatures could result in better chances of recovering from hypothermia or using cold temperatures to preserve organs.

Hypothesis

As hibernation is accomplished with coordinated responses from each tissue, it is important to not only look at the function of each individually, and how they independently respond to the same parameter, but also how they influence the response of other tissues. I hypothesized that respiration rates of BAT mitochondria and adipocytes will be influenced by temperature and hormonal regulators and/or fuels present. These changes were understood by evaluating mitochondrial and brown adipocyte respiration rates. Furthermore, since hormonal regulators and the fuels present depend on the action of other enzymes, I hypothesized that the concentration and availability depends on

season and expression levels of enzymes involved in specific pathways. Measuring the levels of proteins that regulate certain hormones provided insight on how concentrations of regulators or fuels are controlled. Thirteen-lined ground squirrels are constantly in flux preparing for hibernation by consuming a substantial amount of food. During hibernation, they rely exclusively on lipid stores for months, and arousing in the spring much leaner; therefore, it was important to make comparisons across the circannual cycle by using at most six time points: fall (hyperphagic and hypophagic), torpor, IBA, spring, and summer.

CHAPTER 2: Materials and Methods

SECTION 2.1: ANIMALS AND COLLECTION POINTS

Animal Care:

All procedures were approved by and performed in accordance with the University of Minnesota Institutional Animal Care and Use Committee (Protocol #1311-31054A). Wild *Ictidomys tridecemlineatus*, thirteen-lined ground squirrels, were captured via live trapping on private property near Paynesville, Minnesota. Animals were captured by slowly pouring water into burrows and use a butterfly net to contain the animal upon emergence. After capture and transportation to the University of Minnesota Duluth School of Medicine, animals were housed in the AAALAC-accredited Animal Care Facility. Each squirrel was individually kept in plastic top-load cages filled with aspen shavings. Outside of hibernation season (April-October), animals were kept in 12:12 light/dark cycle at 23^{o}C and fed standard rodent chow (Purina #5001) and water *ad libitum*. During the hibernation season (November – March), the squirrels were housed in an artificial hibernation chamber and kept in constant darkness at 5-7^{o}C with water *ad libitum*, but with no food provided.

Experimental Collection Points:

Collection points from across the circannual cycle were used since thirteen-lined ground squirrels are constantly in flux preparing for hibernation by consuming a substantial amount of food, undergoing hibernation where they rely exclusively on lipid stores for months, and finally arousing in the spring much leaner. Because of these changes, four-time points were used for western blots and brown adipocyte assay: fall

active (hyperphagic and hypophagic), torpor, interbout arousal (IBA), spring active. A summer active time point for the mitochondrial assay was also included.

Summer active animals for the mitochondrial assays were caught in the field that spring (May 25th, 2015) and sacrificed between late June and early August of that summer. Some of the summer active squirrels had given birth while in captivity and were collected later to allow for weaning of pups. Throughout the spring and summer, thirteen-lined ground squirrels are giving birth, caring for their pups, and preparing for the upcoming hibernation season by consuming large amounts of food. This hyperphagic characteristic continues into the fall with the highest amounts of daily food consumption occurring between August and September (Schwartz and Andrews, 2013). Hyperphagic squirrels for the studies described were selected based on food consumption; typically, if food consumption was greater than 10 g per day over seven or more days, the squirrel was considered hyperphagic. Hyperphagic sacrifice dates occurred between late August and early October for all studies. Around mid to late October, squirrels drastically lower food consumption to five grams or less per day. Squirrels in this state are sacrificed as hypophagic. Both hyperphagic and hypophagic squirrels were active at the time of collection and were grouped into the fall time point.

By late October, squirrels have typically doubled or even tripled their initial arrival body weight in preparation for the upcoming hibernation season. In addition to nearly stopping all food consumption, the squirrels may undergo shallow torpor bouts where body temperature decreases to room temperature for a several hours. Squirrels were put into the hibernaculum at the end of October/beginning of November to mimic

conditions outside and facilitate typical hibernation patterns. Squirrels from the hibernation time points, torpor and IBA, were collected between December and February when torpor bouts are typically longest (Schwartz and Andrews, 2013). Torpid squirrels were on day four, or later, of a torpor bout and showed no signs of arousal. Squirrels collected for IBA had aroused spontaneously from their torpor bout the previous day and were awake and active.

In April, the squirrels were removed from the hibernation chamber, returned to room temperature, and given food again. Once the squirrels resumed regular food consumption, at least two weeks post removal, they were collected for the spring time point (April – May). All spring animals in these studies completed an entire hibernation season, in the lab, and offer the chance to study how the squirrels recover.

Males and females, a near equal number, were collected at each time point. The state of each animal was determined based on activity level for summer, fall, and spring, or lack thereof for torpid animals, and verified by rectal temperature. Squirrels were deeply anesthetized with 5% isoflurane, verified by toe pinch, and killed via decapitation prior to tissue collection.

Skeletal muscle samples were taken from the quadriceps. The muscles were dissected, cleaned of any contaminating tissue, and flash frozen with liquid nitrogen. Samples were stored at -80^{o}C until protein extraction.

Brown adipose tissue samples were taken from axillary pads. For mitochondrial and adipocyte assays, whole axillary BAT pads were dissected and cleaned of any contaminating tissues before being placed in ice-cold buffer (Sections 2.2 and 2.4).

Brown adipose tissue samples for protein isolation were also taken from the axillary pads. The tissue was dissected and cleaned of any contaminating tissue, flash frozen with liquid nitrogen, and stored at -80^{o}C until protein extraction.

Whole liver lobes were used for mitochondrial assays. Liver lobes were dissected, cleaned of any contaminating tissue, and placed in ice-cold buffer (Section 2.2).

SECTION 2.2: MITOCHONDRIAL ISOLATION

Liver and BAT

Mitochondria were isolated via differential centrifugation by methods adapted and modified from Cannon and Nedergaard, 2008; Li and Graham, 2012; Silva and Oliveria, 2012. Isolation of BAT and liver mitochondria was completed simultaneously, but the tissues and mitochondria were kept in separate containers to prevent mixing.

Immediately following animal sacrifice (Section 2.1), whole liver lobes and both right and left axillary BAT pads were extracted, cleaned of any contaminating tissues, and immediately placed in separate beakers with 30 mL of ice-cold mitochondrial isolation buffer (MIB; ♦1). The tissues were then minced on ice with surgical scissors and homogenized with ten passes in 30 mL of MIB + bovine serum albumin (MIB + BSA; ♦2) using a rotating, loose fitting Teflon pestle. Liver and BAT homogenates were filtered through three layers of sterile gauze and centrifuged at 1,000 *x g* for 10 minutes at 4ºC to remove intact cells, nucleosomes, nuclei, and other cellular tissues. Any floating lipids were aspirated from the supernatants, which were transferred to a clean, pre-chilled centrifuge tubes and centrifuged at 500 *x g* for 10 minutes at 4 ºC removing any additional fatty cells that were not previously removed. Any floating lipids were aspirated from the supernatants, which were transferred to clean, pre-chilled centrifuge tubes and centrifuged at 10,500 *x g* for 10 minutes at 4 ºC to form pellets containing mitochondria. The supernatants were decanted and any lipid adhering to the tubes were removed with KimWipes. The pellets were re-suspended in 30 mL ice-cold wash buffer + BSA (WB + BSA; ♦3) and centrifuged at 12,000 *x g* for 10 minutes at 4ºC. The

supernatants were decanted and the mitochondrial pellets were re-suspended in 30 mL ice-cold wash buffer (WB; ♦4) and centrifuged at 12,000 *x g* for 10 minutes at 4ºC. The final mitochondrial pellets were transferred to pre-chilled Eppendorf tubes and kept on ice until assayed for mitochondrial respiration (maximum 6 hours).

The isolated mitochondrial protein concentrations were determined using bicinchoninic acid (BCA) protein assay (Pierce BCA assay – Thermo Scientific, #23255, 23277) per the manufacturer's protocol with BSA as a standard. The protein determination was used to determine the volume of mitochondria to use for a final concentration of 0.5 mg protein/mL. Mitochondrial respiration measurements were performed directly after the mitochondrial isolation starting at 5ºC.

SECTION 2.3: *IN VITRO* MITOCHONDRIAL RESPIRATION

Clark-type Oxygen Electrode:

A Clark-type oxygen electrode is commonly used for investigation of the proton circuit in mitochondria measuring the concluding transfer of electrons from the ETS to O_2 (Nicholls and Ferguson, 2013). Using a Clark-type oxygen electrode, multiple reagents, and isolated mitochondria can be added during an experiment for analysis of different activity (Nicholls and Ferguson, 2013). A Clark-type oxygen electrode uses two electrodes, a platinum and silver reference electrode, and the current flowing between the two is measured which is proportional to the oxygen consumption (Nicholls and Ferguson, 2013). The solution is constantly stirred to prevent an anoxic layer from forming on the surface of the oxygen permeable membrane covering the electrodes, thus interfering with an accurate measurement (Nicholls and Ferguson, 2013).

The Clark-type oxygen electrode from Hansatech Instruments was used for all oxygen consumption measurements. Depending on the temperature, the concentration of gasses dissolved in solution varies, therefore, the oxygen electrode was equilibrated at each temperature under both air saturating and anoxic conditions in 1 mL of dH_2O with constant stirring. Air saturated measurement was completed first by allowing the oxygen electrode to plateau at 100% oxygen. After the plateau was reached, a pinch of dithionite was added to the chamber and allowed to reach a plateau of 0% oxygen. The calibration was then saved and able to be used continuously at the set temperature for up to 24 hours.

Isolated liver and BAT mitochondrial respiration measurement

Mitochondrial respiration rates from liver and BAT were measured separately *in vitro* using a Clark-type oxygen electrode (Hansatech Instruments) at 5, 13, 21, 29, and 37^{o}C in 0.5 or 1 mL of respiration buffer (♦5) with constant stirring. A range of temperatures was used to mimic an arousal from torpor to IBA. Reagents were dissolved in either dH_2O (succinate, glutamate, and ADP) or ethanol (rotenone) and added to the chamber using Hamilton syringes.

Liver and BAT mitochondria were added to a final concentration of 0.5 mg/mL. Maximal flux through Complex II was measured under *state 4* (non-phosphorylating, BAT) and *state 3* (phosphorylating, liver) conditions with the addition of ADP (200 nM) (Chance and Williams, 1955). Before the addition of the substrate succinate (5 mM), rotenone (2mM) was added to prevent reverse electron flow and the contribution of Complex I to respiration rates (Nicholls and Ferguson, 2013). Glutamate (5 mM) was added to relieve Complex II inhibition by oxaloacetate (Staples, 2014) before the addition of ADP (200 nM). Some BAT spring temperature measurements may have low sample sizes due to not obtaining many mitochondria. Once all assays were complete, isolated mitochondria were stored at -80^{o}C.

SECTION 2.4: ISOLATION OF BAT AND SKELETAL MUSCLE PROTEIN

Axillary BAT pads and quadriceps muscles from ground squirrels were extracted, cleaned of any contaminating tissues, and flash frozen at -80ºC in liquid nitrogen until extraction of protein. Tissue samples were isolated from four-time points across the circannual cycle; fall (hyperphagic and hypophagic), torpor, IBA, and spring active.

To extract protein, radioimmunoprecipitation assay buffer (RIPA; ♦6) with protease and phosphatase inhibitors (RIPA I^2) was prepared by addition of 1 µL per mL protease inhibitor cocktail, phosphatase inhibitor cocktail 2, and phosphatase inhibitor cocktail 3 to enough RIPA for the number of extractions (about 0.5 mL per sample). Whole tissue isolates were placed in liquid nitrogen and crushed using a pre-frozen (in liquid nitrogen) mortar and pestle. Approximately 0.05 – 0.1 g of tissue was placed in a 2-mL round bottom tube. A 10% homogenate solution was made on a mass to volume basis by adding a volume of RIPA I^2 that equaled nine times the mass of the tissue sample. The sample was thawed in RIPA I^2 before homogenization with a Tissue tearor® motor-driven homogenizer at a medium speed until no tissue chunks remain or for a maximum of 1 minute. After homogenization, the sample was placed on ice for 30 minutes with periodic vortexing to allow for inhibitor action, lysis, and extraction of protein from cells. The sample was then centrifuged at 10,000 *x* g for 20 minutes at 4ºC to get rid of cell debris and nucleus.

The isolated protein concentrations were determined using BCA protein assay (Pierce BCA assay – Thermo Scientific #23255, 23277) per the manufacturer's protocol with BSA in 0.1X RIPA as a standard. The supernatant from the 10,000 *x* g

centrifugation was transferred to a new 1.5 mL tube and diluted 1:10 with water before protein determination. The protein concentration was used to determine the volume of muscle homogenate to be loaded for Western blot for a final concentration of 2 μg/μL, after dilution in Laemmli sample buffer (LSB; ♦7). Once diluted in LSB, samples were incubated at 90ºC for 10 minutes after which samples were either used or stored by freezing at -80ºC until used.

SECTION 2.5: WESTERN BLOTTING

Adapted from Bio-Rad and Laemmli, U.K., 1970

Protein samples, 40 µg/lane were loaded on a 10% SDS-PAGE mini gel and separated by electrophoresis at approximately 175 V for 1 hour in running buffer (Running Buffer; ♦8). Proteins were then transferred onto a nitrocellulose membrane in Tris-glycine buffer (Transfer Buffer; ♦9) with 20% methanol at 75 V for 3 hours. To evaluate completeness of transfer and verify equal protein loading, the membranes were stained with Ponceau S (P7170; Sigma). Membranes were destained with two washes of Tris Buffered Saline with 0.1% (by volume) Tween 20 (TBST; ♦10) for 10 minutes each while undergoing constant shaking and then blocked with 3% BSA in TBST for 3 hours at room temperature while undergoing constant shaking. Membranes were incubated with the primary antibody (WB antibodies; ♦11) diluted in TBST with 0.3% BSA at 4ºC overnight while undergoing constant shaking. Membranes were then rinsed with three, 10 minute washes with approximately 30 mL TBST before incubation with secondary antibody, diluted 1: 10,000 in TBST, for 1 hour at room temperature with constant shaking. The secondary antibody was removed with three, 10 minute washes with approximately 30 mL TBST. Reactive protein was detected by chemiluminescence using SuperSignal West Pico (ThermoFisher Scientific; #34080). After a 5-minute incubation period, the protein was imaged and analyzed using LI-COR Odyssey® FC. A pooled control containing one sample from each time point was included on all membranes and was used to normalize protein amounts between blots.

SECTION 2.6: ISOLATION OF BROWN ADIPOCYTES

Brown adipocyte isolation methods were adapted from Cannon and Nedergaard, 2008; McFarlane et al, 2017. Immediately following animal sacrifice (Section 2.1) both right and left axillary BAT pads were extracted, cleaned of any contaminating tissues, and placed in a beaker with 3 mL of ice-cold Krebs/Ringer Phosphate Buffer (KR; ♦12). The tissue was then incubated in 10 mL KR with collagenase (KR + C; ♦13) for 5 minutes in a shaking water bath at 37ºC after which, the tissue was vortexed for 5 seconds and filtered onto 250 µm nylon mesh. The tissue was then minced with surgical scissors and incubated in 3 mL KR + C at 37ºC for 25 minutes with vortexing for 5 seconds every 5 minutes. Seven mL of KR + C was then added, the tissue was vortexed for 15 seconds, and the filtrated was collected and placed on ice after filtration through 250 µm nylon mesh. Any tissue pieces left were incubated again in 3 mL KR + C for 15 minutes at 37ºC, with vortexing for 5 seconds every 5 minutes. After, 7 mL of KR + C was added to the tissue and vortexed for 15 seconds. The tissue was filtered again through 250 µm nylon mesh, the filtrate was collected and placed on ice. To increase yield, any tissue pieces remaining on the nylon mesh were collected and incubated again in 3 mL of KR + C for 15 minutes in a shaking water bath at 37ºC with vortexing every 5 minutes for 5 seconds. Following the 15-minute incubation, 7 mL KR + C was added and the tissue was vortexed 15 seconds, filtered through 250 µm nylon mesh and the filtrate was collected. All filtrates were combined and centrifuged at 76 *x g* for 5 minutes at 4ºC to separate lipids, stromal-vascular cells, pre-adipocytes, red blood cells, and other tissue fragments. The solution was then placed on ice for 30 minutes for separation of

brown adipocytes. The cells were then collected from the adipocyte layer using a Pasteur pipette and bulb. Isolated cells were stored on ice until assayed (maximum of 4 hours). Before being assayed, cell concentration was determined using a hemocytometer with viability determined by trypan blue (0.4% trypan blue solution; Sigma-Aldrich) staining. After assay completion, remaining cells were stored at -80ºC.

SECTION 2.7: MEASUREMENT OF BROWN ADIPOCYTE RESPIRATION RATES

Respiration rates of isolated brown adipocytes were measured using a Clark-type oxygen electrode (Hansatech Instruments) that was calibrated as described previously (Section 2.3). Respiration assays were carried out in 1 mL of Minimal Dulbecco's Modified Eagle Medium supplemented with pyruvate, L-glutamine, glucose, and HEPES (DMEM; ♦14) and performed at 10 and 37^{o}C, with calibration at each temperature, and while undergoing constant stirring. These temperatures were chosen to evaluate both low and high body temperatures thirteen-lined ground squirrels reach throughout their circannual cycle. Cells were added to the chamber at a final concentration of 650,000 cells/mL in DMEM and the basal respiration rate was recorded for three minutes. To evaluate the effect of adenosine on brown adipocyte respiration, β-adrenergic agonist isoproterenol (1 mM) was first added to stimulate respiration. Cellular respiration with isoproterenol present was measured after a three-minute incubation period before the addition of adenosine (0.5 mM). Cellular respiration in the presence of adenosine was measured three minutes after adenosine addition. To see if the effect of adenosine could be reversed, another addition of isoproterenol (1.5 mM) was made after a respiration rate with adenosine present was recorded.

SECTION 2.8: DATA ANALYSIS

JMP Pro 12 statistical software was used for all statistical analyses. Data is presented as means ± standard error (SE) and data was considered statistically significant when $P < 0.05$.

Mitochondrial respiration

Significant differences in liver and BAT mitochondrial respiration rates between season and temperature were determined using a two-way analysis of variance (ANOVA). Significant differences were further analyzed using a Tukey's highly significant difference (HSD) test to find which means were statistically different. Using a two-way ANOVA allows for comparisons between two factors, in this case temperature and season, and how both factors impact the mean respiration rate. Following a two-way ANOVA with Tukey's test identifies which groups (or seasons) are different from others at each temperature.

Western blotting

Significant differences in protein expression between seasons were determined using a one-way ANOVA. Significant differences were further analyzed using a Tukey's HSD test to find which means were statistically different. Using ANOVA for statistical analysis allows comparisons of the means from multiple groups (or seasons) and identifies whether there are significant differences. Furthermore, an ANOVA finds the size of the difference and provides a degree of certainty regarding how different the

groups are. Following an ANOVA test with Tukey's test identifies which groups (or seasons) are different from others.

Adipocyte respiration

Significant differences in adipocyte respiration rates between season and substrates present were determined using a split-plot ANOVA. Significant differences were further analyzed using a Tukey's HSD test to find which means were statistically different. A two-way ANOVA was an appropriate test for this data set because it allows groups to be subdivided and accounts for repeated measures with random effects. Therefore, the response variable (respiration rate) depends on several variables (substrate and season), but also accounts for the random variability that occurs (different squirrels randomly selected from a population whose adipocyte respiration rate was measured more than once). Following a two-way ANOVA with Tukey's allows for identification of which groups (seasons and substrates) are different from the others.

SECTION 2.9: SOLUTIONS INDEX

♦1 Mitochondrial Isolation Buffer (MIB)

250 mM Sucrose
5 mM HEPES
1 mM EGTA
pH ~ 7.2-7.4 (using KOH)
Store and use at 4ºC

♦2 Mitochondrial Isolation Buffer + Bovine Serum Albumin (MIB + BSA)

250 mM Sucrose
5 mM HEPES
1 mM EGTA
0.1% fatty acid free bovine serum albumin (BSA)
pH ~ 7.2-7.4 (using KOH)
Store and use at 4ºC

♦3 Wash Buffer + Bovine Serum Albumin (WB + BSA)

250 mM Sucrose
5 mM HEPES
0.1% fatty acid free bovine serum albumin (BSA)
pH ~ 7.2-7.4 (using KOH)
Store and use at 4ºC

♦4 Wash Buffer

250 mM Sucrose
5 mM HEPES
pH ~ 7.2-7.4 (using KOH)
Store and use at 4ºC

♦5 Respiration Buffer

135 mM Sucrose
65 mM KCl
5 mM KH_2PO_4
5 mM HEPES
2.5 mM $MgCl_2$
250 mM Sucrose
5 mM HEPES
pH ~ 7.2-7.4 (using KOH)

Store and use at 4°C

♦6 Radioimmunoprecipitation Assay Buffer (RIPA

150 Mm NaCl
1.0% Triton X-100
0.5% Sodium deoxycholate
0.1% Sodium dodecyl sulfate
50 mM Tris
pH ~ 8.0
Store at -20°C

Protease inhibitor cocktail P8340
Phosphatase inhibitor cocktail 2 P5729
Phosphatase inhibitor cocktail 3 P0044

♦7 Laemmli Sample Buffer, 5X (LSB)

From Kirschner Lab, online (10 mL)
2.5 mL of 1.25 M Tris (pH 6.8)
5 mL of 100% Glycerol
1g Sodium dodecyl sulfate
0.8g Dithiothreitol
1 mL Bromophenol Blue (1% solution)
Q.S. to 10 mL with dH_2O

♦8 Running Buffer (RB)

10X Stock:
250 mM Tris Base
1.9 M Glycine
10 g Sodium dodecyl sulfate
Q.S. to 1L with dH_2O
Store at room temperature

Use as 1X Running Buffer

♦9 Transfer Buffer (TB)

10X Stock:
250 mM Tris Base
1.9 M Glycine
Q.S. to 1L with dH_2O
Store at 4°C

Use as 1X Transfer Buffer
With 20% Methanol

♦10 Tris Buffered Saline + Tween 20 (TBST)
10X TBS stock:
0.5 M Tris Base
1.5 M NaCl
pH ~ 7.5 (with HCl)
Q.S. to 1L with dH_2O
Store at room temperature

Use at 1X TBS
With 0.1% (Vol: Vol) Tween 20

♦11 Western Blotting Antibodies

All antibodies were added to 10 mL of 0.3% Bovine Serum Albumin in Tris Buffered Saline with 0.1% Tween 20 (BSA in TBST)

Primary Antibodies

- *AMPD1* (ab72541) 1:2000
- *SLC29A1* (ab135756) 1:2000
- *AMPK* (ab3760) 1:2000
- *AMPK*-Phosphorylated (Phospho T183 alpha 1, Phospho T172 alpha 2, ab195946) 1:2000
- *ADORA1* (ab75177) 1:1000

Secondary Antibody (31460, Goat anti-Rabbit IgG, HRP)
1:10000

♦12 Krebs – Ringer Phosphate Buffer (KR)

109 mM NaCl
6.9 mM KCl
1.5 mM $CaCl_2$
1.4 mM $MgSO_4$
1.7 mM Na_2HPO_4
5.6 mM NaH_2PO_4
20 mM Glucose
4% Fatty Acid Free Bovine Serum Albumin
pH ~ 7.2-7.4 (using KOH)

♦13 Krebs - Ringer Phosphate Buffer + Collagenase (KR + C)

109 mM NaCl
6.9 mM KCl
1.5 mM $CaCl_2$
1.4 mM $MgSO_4$
1.7 mM Na_2HPO_4
5.6 mM NaH_2PO_4
20 mM Glucose
4% Fatty Acid Free Bovine Serum Albumin
1.66 mg/mL Type II Collagenase
pH ~ 7.2-7.4 (using KOH)

♦14 Minimal Dulbecco's Modified Eagle Medium (supplemented with pyruvate, L-glutamine, glucose, and HEPES) (DMEM)

Minimal Dulbecco's Modified Eagle Medium
Supplement with the following the day of the experiment
1 mM Sodium Pyruvate
2 mM L-Glutamine
10 mM Glucose
5 mM HEPES
pH ~ 7.4
Store and use at 4°C

CHAPTER 3: Temperature Effect on Mitochondrial Respiration

∗ This chapter was included in the following manuscript, which was published in the American Journal of Physiology – Regulatory, Integrative, and Comparative Physiology: Ballinger, M.A., Hess, C., Napolitano, M.W., Bjork, J.A., and Andrews, M.T. (2016) Interrogation of Brown Adipose Tissue Mitochondria in a Mammalian Hibernator: from Gene Expression to Function. 311 (2): R325-R336.

SECTION 3.1: RESULTS

To assess temperature effect on mitochondrial respiration rates of thirteen-lined ground squirrels, liver and BAT mitochondria were isolated and succinate-fueled respiration rates in the presence of ADP were measured at five temperatures (5, 13, 21, 29, and 37^{o}C). Mitochondria were isolated from liver and BAT samples from five time points throughout the circannual cycle: summer (SUM), spring (SP), fall (FALL), torpor (TOR), and interbout arousal (IBA). In BAT, only one significant difference was found at 5^{o}C with summer respiration rates being significantly lower than torpor (Figure 3.1A) while in liver, at least one significant difference was found at each temperature and between each season (Figure 3.1B; Table 3.1).

Liver and BAT Mitochondrial Respiration

Electron transport system complexes from BAT mitochondria of thirteen-lined ground squirrels were individually examined at 25^{o}C across the circannual cycle by *Ballinger et al* (2016). Using a series of fuels and inhibitors, no significant differences were found at Complexes I and IV. However, at Complex II fueled by succinate, spring mitochondria had significantly lower respiration rates than fall, torpor, and IBA. At Complex III, fueled by glycerol-3-phosphate (G3P), mitochondria isolated from squirrels in IBA had significantly higher respiration rates than mitochondria isolated from fall squirrels. Respiration rates at Complexes II-IV were higher, although non-significantly, throughout hibernation and no significant differences were found between torpor and IBA at any complex.

Based on the *Ballinger et al* (2016) findings, studies were completed to see how temperature affects respiration rates of BAT and liver, as a non-thermogenic tissue comparison, mitochondria fueling at Complex II with succinate across the circannual cycle. We hypothesized that respiration rates would be greater in BAT mitochondria compared to liver and the differences in these rates would not be as great in BAT regardless of season, unlike liver mitochondria. Mitochondrial respiration rates at each temperature and across the five time points were determined via Clark-type oxygen electrode from Hansatech Instruments and statistically analyzed by two-way ANOVA followed by Tukey's HSD test to identify which groups (or seasons) were different from others at each temperature (Section 2.8). Mitochondrial respiration rates were considered statistically different when $P < 0.05$.

Results show that isolated liver mitochondria are more sensitive to temperature and season than BAT. In BAT, only one significant difference was found; at 5^{o}C, torpor respiration rates were significantly higher than summer ($P = 0.0341$, Figure 3.1A). No other significant differences were found between all seasons (SP, SUM, FALL, TOR, and IBA) at all temperatures (5, 13, 21, 29, and 37^{o}C (Figure 3.1A). Liver, however, showed greater sensitivity to temperature across the circannual cycle (Figure 3.1B; Table 3.1). When comparing respiration rates, torpor rates were significantly lower than all other seasons at three or more temperatures ($P <0.0001$; Figure 3.1B; Table 3.1). Specifically, torpor was significantly lower than summer at all five temperatures, fall at four temperatures (13, 21, 29, and 37^{o}C), and spring and IBA at three temperatures (13, 21,

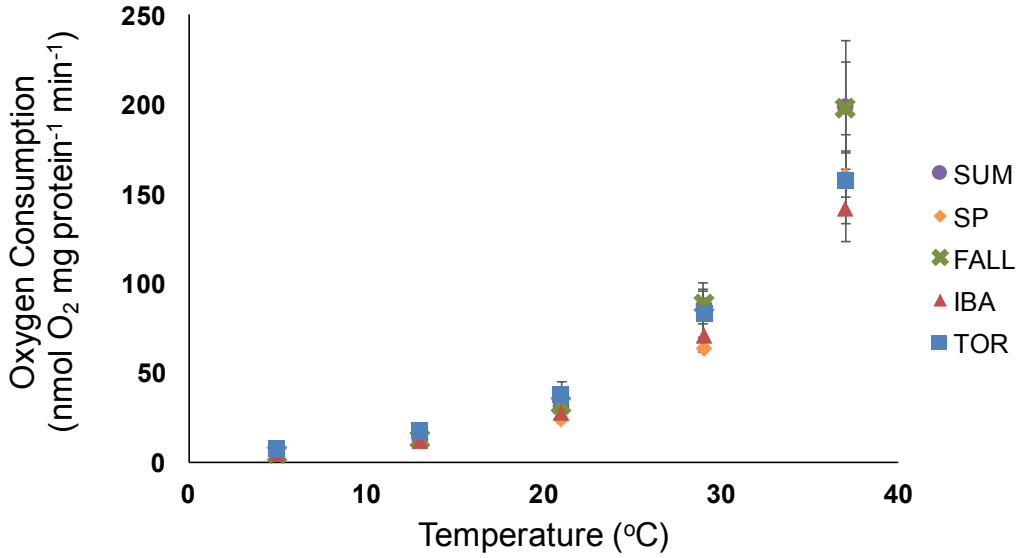
and 29°C (P <0.05, Figure 3.1B and Table 3.1). In both BAT and liver, respiration rates at 5°C are significantly lower than rates at 37°C (P <0.0001, Figures 3.1A, B).

Additional comparisons were performed to understand whether decreases in metabolism are due to passive thermal effects or whether metabolism is actively inhibited using a Q_{10} analysis. Temperature coefficients (Q_{10}) reflect the capacity of organisms to change their metabolic rate relative to changes in temperature where the lower four temperatures (5, 13, 21, and 29°C) were set at T_1 and each was compared to 37°C, set at T_2 (Baust and Baust, 2007; Geiser, 1988; Staples, 2014). The second rate measurement (R_2) corresponded to the average respiration rate at 37°C and the respiration rate corresponding to the four other temperatures (5, 13, 21, and 29°C) were set at R_1. The Q_{10} equation is: $Q_{10} = (R_2 / R_1) \text{ ^ } (10 / (T_2 – T_1))$. Based on these comparisons, most BAT and liver Q_{10} values fall between 2 and 3 (Table 3.2), suggesting passive thermal effects play a major role in reducing metabolism (Guppy and Withers, 1999; Snapp and Heller, 1981; Staples and Brown, 2008). When Q_{10} is greater than 3, active processes also regulate metabolic suppression (Geiser, 1988; Staples and Brown, 2008). For both BAT and liver, higher Q_{10} values were typically obtained at lower temperatures with a trend toward decreasing Q_{10} values as the temperature approached 37°C (Table 3.2). In BAT, Q_{10} values were only available for two comparisons; 5 and 13°C both compared to 37°C.

Section 3.1 Figures and Tables

Figure 3.1. Temperature effects of respiration rates in isolated BAT and liver mitochondria. *Significant differences between two or more season at any given temperature given by Tukey's highly significant difference test. A) Respiration rates of BAT mitochondria at different temperatures. State 4 respiration rates were measured at succinate dehydrogenase (complex II), using succinate as a fuel at five different temperatures (5, 13, 21, 29, and 37ºC). Data are expressed as means ± SE; FALL n = 8, TOR n = 8, IBA n = 10, SP n = 8 at 5ºC and 37ºC, n = 7 at 13ºC, n = 3 at 21ºC and 29ºC, and SUM n = 10. B) Respiration rates of liver mitochondria at different temperatures. State 3 (phosphorylating) respiration rates were measured at succinate dehydrogenase (complex II), using succinate as a fuel at five different temperatures (5, 13, 21, 29, and 37ºC). Data are expressed as means ± SE; FALL n = 8, TOR n = 8, IBA n = 13, SP n = 8, SUM n = 10. Abbreviations: SUM, summer; SP, spring; IBA, interbout arousal; TOR, torpor.

A.



B.

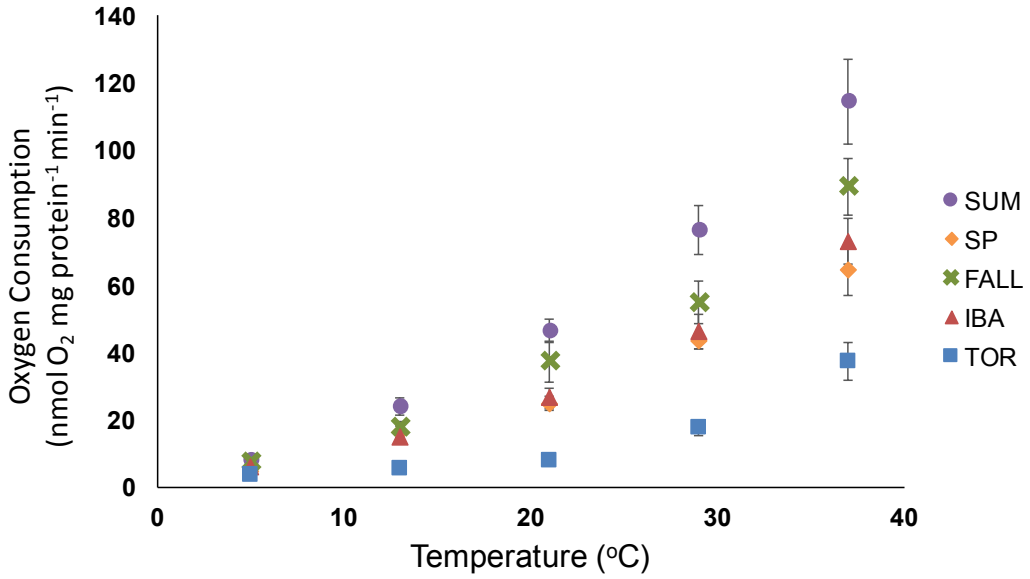

Oxygen Consumption
(nmol O_2 mg protein^{-1} min^{-1})
140
120
100
80
60
40
20
0
0
10
20
30
40
Temperature (°C)
SUM
SP
FALL
IBA
TOR

Table 3.1. Significant differences in respiration rates of liver mitochondria between seasons at each temperature measured (in ^{o}C). Based on respiration rates of liver mitochondria at different temperatures (5, 13, 21, 29, and 37^{o}C). State 3 (phosphorylating) respiration rates were measured at succinate dehydrogenase (complex II), using succinate as a fuel. Significant differences determined by P <0.05 from two-way ANOVA. Abbreviations: IBA; interbout arousal; (NS), no significant differences.

	TORPOR	IBA	FALL	SUMMER
SPRING	13^{o}, 21^{o}, 29^{o}	(NS)	(NS)	21^{o}, 29^{o}, 37^{o}
SUMMER	5^{o}, 13^{o}, 21^{o}, 29^{o}, 37^{o}	13^{o}, 21^{o}, 29^{o}, 37^{o}	(NS)	
FALL	13^{o}, 21^{o}, 29^{o}, 37^{o}	(NS)		
IBA	13^{o}, 21^{o}, 29^{o}			

Table 3.2. Mean respiration rate represented as a Q_{10} comparison for A) BAT mitochondria and B) liver mitochondria across the circannual cycle for a temperature range of 37°C to 5°C. Temperature coefficients (Q_{10}) values reflect the capacity of organisms to change their metabolic rate relative to changes in temperature (Foss et al, 2011 and Staples, 2014). Each temperature (5, 13, 21, and 29°C = T_1) is compared to 37°C. The second temperature (T_2) was set at 37°C and the second rate measurement (R_2) corresponded to the average respiration rate at 37°C. The respiration rate corresponding to the four other temperatures (5, 13, 21, and 29°C) were set at R_1. The Q_{10} equation is: $Q_{10} = (R_2 / R_1) \wedge (10 / (T_2 - T_1))$ (Baust and Baust, 2007). Abbreviations: SUM, summer; SP, spring; FALL, fall; IBA, interbout arousal; TOR, torpor.

A.

BAT	Q_{10} (37°C - 5°C)	Q_{10} (37°C - 13°C)	Q_{10} (37°C - 21°C)	Q_{10} (37°C - 29°C)
SUM	3.22	3.09	3.13	2.8
SP	2.97	2.67	N/A	N/A
FALL	3.18	3.12	3.12	2.73
IBA	2.8	2.77	2.75	2.37
TOR	2.55	2.47	2.42	2.22

B.

LIVER	Q_{10} (37°C - 5°C)	Q_{10} (37°C - 13°C)	Q_{10} (37°C - 21°C)	Q_{10} (37°C to 29°C)
SUM	2.28	1.92	1.76	1.66
SP	2.28	1.76	1.81	1.64
FALL	2.17	1.96	1.72	1.84
IBA	2.17	1.93	1.89	1.77
TOR	2.1	2.22	2.69	2.54

SECTION 3.2: DISCUSSION

Brown adipose tissue functions to facilitate whole organism re-warming via non-shivering thermogenesis. This rewarming is particularly important as squirrels arouse from torpor to IBA at regular intervals throughout hibernation. Rewarming via non-shivering thermogenesis is primarily accomplished through the action of BAT mitochondria and in particular, UCP1 that uncouples the ETS from ATP synthesis and dissipates the proton gradient producing heat instead of energy (ATP). Furthermore, BAT functions while most other tissues are relatively inactive. Liver function accounts for approximately 20% of the basal metabolic rate during the active state (van Breukelen and Martin, 2001); therefore, by suppressing activity during hibernation, a substantial amount of energy and heat, produced as a metabolic byproduct, is conserved. Suppression of mitochondrial respiration has been studied previously in other tissues including liver, heart, and skeletal muscle (Barger et al, 2003; Brown et al, 2012; Brustovetsky et al, 1989; Muleme et al, 2006) focusing mainly on the transition between torpor and IBA. These studies found varying degrees of suppression depending on the tissue, but significant differences were observed with depressed respiration rates occurring during torpor compared to other seasons (Barger et al, 2003; Brown et al, 2012; Brustovetsky et al, 1989; Muleme et al, 2006).

The present study examined what influence temperature and season have on isolated mitochondrial respiration rates from thermogenic BAT and non-thermogenic liver from thirteen-lined ground squirrels. When fueled with succinate, we predicted BAT mitochondrial respiration rates would be less sensitive to temperature compared to

liver mitochondria and that time of the year would have less of an effect on BAT mitochondria than liver. To test these hypotheses, we measured succinate-fueled oxygen consumption rates of mitochondria isolated from five seasonal time points: fall (FALL), spring (SP), summer (SUM), torpor (TOR), and interbout arousal (IBA). The isolated mitochondria were assayed at five temperatures (5, 13, 21, 29, and 37ºC).

Liver mitochondria show seasonal and temperature sensitivity

Results show that liver mitochondria are more sensitive to both assay temperature and season than BAT mitochondria. In liver, *state 3* (phosphorylating) mitochondrial respiration rates from torpid squirrels were significantly lower than all other seasons at three or more temperatures (Figure 3.1B; Table 3.1). Furthermore, torpor was significantly lower than summer at all five temperatures, fall at four temperatures, and spring and IBA at three temperatures (Figure 3.1B; Table 3.1). Overall, liver respiration rates at 5ºC are significantly lower than rates at 37ºC (Figures 3.1B).

Furthermore, respiration rates and Q_{10} results suggest liver mitochondria experience passive and active regulated changes to lower activity during torpor. Another study examining temperature impact on liver mitochondrial respiration rates fueled by succinate found similar differences that we did when comparing torpor, IBA, and summer respiration rates at 5, 25, and 37ºC (Muleme et al, 2006). However, *Muleme et al* (2006) did not find any significant differences at 5ºC. Comparing the same seasons, summer and torpor, at 5ºC we found that torpor respiration rates were significantly lower than summer. The discrepancies in the results could be due to sampling periods between our

study and *Muleme et al* (2006). *Muleme et al* (2006) used May to early August as their summer time point while we used April-May as our spring collection point and late June to early August as summer. Nevertheless, both studies suggest liver mitochondria are sensitive to both temperature and season throughout the year, with torpor respiration being the most sensitive.

Our study focused on the activity of ETS downstream of Complex I (Complex II and beyond), the activity of citric acid cycle enzymes, and the activity of dicarboxylate transporter, SLC25A10, which functions to transport succinate into the mitochondrial matrix. Temperature sensitivities and changes in protein levels could influence respiration rates of mitochondria. Some citric acid cycle enzymes are temperature sensitive (Carey et al, 2003) and mRNA expression of *SLC25A10* are unknown in the liver, but differentially expressed in heart and cortex. In most tissues, including heart and cortex, *SLC25A10* levels are higher in torpor and IBA than October and April.

Additionally, liver mitochondrial Complex II activity was lower in torpor squirrels compared to IBA when measured at 37^{o}C and with varying concentrations of succinate, but this same pattern was not evident when Complex II activity was measured at 10^{o}C (Brown et al, 2013). Maximal activity of Complex II was suppressed in both freeze-thaw isolated liver mitochondria and homogenized liver samples between torpor and IBA (Mathers et al, 2016). However, there were no changes in the protein content of Complex II between torpor and IBA (Mathers et al, 2016). The *Mathers et al* (2016) results, coupled with our measurement of liver mitochondrial respiration, suggests that changes at Complex II, such as inhibition possibly by citric acid cycle intermediate

oxaloacetate, could contribute to the initial depressed liver mitochondrial respiration upon entrance into torpor when body temperature is still decreasing (Brown et al, 2013; Staples, 2014; Armstrong and Staples, 2010). Also, there could be post-translational modifications occurring on Complex II that are rapid and reversible for example deacetylation, phosphorylation, and desuccinylation (succinyl group is removed from a lysine residue, Mathers et al, 2016).

Passive thermal effects account for most of the decrease in respiration rates in liver and BAT

In addition to Complex II activity inhibition, as well as potential inhibition of other ETS enzymes, there could be other mechanisms in place reducing liver mitochondrial function that displays as reduced respiration rates, such as substrate availability for mitochondrial function and temperature sensitivity. The respiration rate, or rate oxygen is consumed, in mitochondria can be used as a measure of function (Baust and Baust, 2007; Geiser et al, 2014). To compare reaction rates, the Q_{10} temperature coefficient can be used to demonstrate how dependent a rate of reaction is on temperature (Baust and Baust, 2007). Temperature dependence could also change depending on fuel utilization since enzymes of the citric acid cycle are not as efficient at low temperatures and fatty acids are more abundant fuel source during hibernation (Carey et al, 2003; Cannon and Nedergaard, 2004), therefore fueling mitochondria with different substrates may change Q_{10} results. Q_{10} has still been applied to several other hibernation studies

considering temperature changes for assays in various tissues (McFarlane et al, 2017; Geiser, 1988; Brown et al, 2012; Muleme et al, 2006).

Based on Q_{10} comparisons, most BAT and liver values fall between 2 and 3, which is typical for most enzyme catalyzed reactions (Table 3.2), suggesting temperature alone accounts for the observed declines in metabolic rate (Guppy and Withers, 1999; Snapp and Heller, 1981; Staples and Brown, 2008). When Q_{10} is greater than 3, active processes also regulate metabolic suppression (Geiser, 1988; Staples and Brown, 2008). For both BAT and liver, higher Q_{10} values were typically obtained at lower temperatures with a trend toward decreasing Q_{10} values as the temperature approached 37ºC (Table 3.2A and B). More specifically, liver Q_{10} values were below 3 regardless of season and temperature range, suggesting that passive thermal effects account for much of the suppression observed (Table 3.2B).

In BAT, Q_{10} values were only available for two comparisons; 5 and 13ºC both compared to 37ºC (Table 3.2A). Brown adipose tissue mitochondria with Q_{10} values greater than 3 were observed from summer and fall animals at the three lowest temperatures (5, 13, and 21ºC; Table 3.2A). These results suggest temperature alone cannot account for the depressed respiration rates and therefore, there may be a greater reliance on active processes for function at these lower temperatures. There could be some intrinsic regulation on BAT mitochondria present throughout summer and fall that would allow this function to remain the same across the circannual cycle. Seasonal differences in regulation could occur at the enzyme level, such as citric acid cycle or ETS, or structural protein level, such as membrane transporters. Fuel selection for

mitochondrial respiration could also change Q_{10} results. A recent study by *McFarlane et al* (2017) measured Q_{10} values from torpor and IBA mitochondrial respiration rates in thirteen-lined ground squirrels at 10 and 37^{o}C fueled with pyruvate and fatty acid octanoyl carnitine. Comparable to our study, Q_{10} values were lower from torpor squirrels compared to IBA. However, the mean values obtained were also lower than those obtained from succinate-fueled respiration (McFarlane et al, 2017) which could suggest fuel differences. Unfortunately, *McFarlane et al* (2017) did not look at any other seasons or temperatures making it impossible to see if the same trend is exhibited across the circannual cycle and therefore lend insight on the impact of fuel selection on Q_{10} values.

BAT mitochondrial metabolic activity is relatively insensitive to temperature and season

Current research on maximal respiration rates of uncoupled BAT mitochondria isolated from thirteen-lined ground squirrels suggests that there is an overall decrease in mitochondrial respiration outside the hibernation season (Ballinger et al, 2016). Specifically, there is no significant difference between torpor and IBA respiration rates at 25^{o}C when each complex is fueled separately (Ballinger et al, 2016). When compared across the circannual cycle, only Complex II resulted in significantly different mitochondrial respiration rates between spring active and hibernation, but only when measured at 25^{o}C (Ballinger et al, 2016).

Unlike succinate-fueled respiration rates from liver mitochondria, only one significant difference was observed in BAT across all temperatures and seasons. In BAT, *state 4* (non-phosphorylating) torpor respiration rates were significantly higher than

summer when measured at 5ºC (Figure 3.1A). Although no other significant differences were observed, torpor respiration rates were higher than all other seasons when measured at the three lowest temperatures (5, 13, and 21ºC; Figure 3.1A). At the two highest temperatures (29 and 37ºC), fall and summer respiration rates were greatest (Figure 3.1A). These results, coupled with the measurements at 25ºC, suggest BAT has an increased respiratory capacity during hibernation which could help facilitate whole organismal rewarming via non-shivering thermogenesis during arousal from torpor to IBA. Additionally, measurement of ETS enzyme activities were not significantly different between torpor and IBA when measured at 10 and 37ºC (McFarlane et al, 2017). This suggests that any suppression that occurs in BAT mitochondria is not occurring on the enzymes of the ETS; however, other interactions at the cellular level, or with other proteins could be occurring.

Since fatty acids are the preferred substrate for BAT mitochondria, respiration rates with fatty acids are thought to be higher than those with other substrates like α-glycerol phosphate (Prusiner et al, 1968) or succinate. The study by *McFarlane et al* (2017) used pyruvate and fatty acid octanoyl carnitine as substrates for measurement of mitochondrial respiration rates at 10 and 37ºC from thirteen-lined ground squirrels throughout hibernation (torpor and IBA only). Results show a significant difference in respiration rates between torpor and IBA animals when measured at 37ºC fueled by both pyruvate and octanoyl carnitine (McFarlane et al, 2017). At 10ºC, no significant differences in respiration rates were observed (McFarlane et al, 2017). Furthermore, IBA rates were higher than torpor rates when measured at 37ºC, but the opposite held true

when measured at 10ºC (McFarlane et al, 2017). However, this study only compared mitochondrial respiration rates from torpor and IBA squirrels at two temperatures, 10 and 37ºC.

Overall, the *McFarlane et al* (2017) study demonstrates respiration rates due to fatty acid oxidation, citric acid cycle, and all components of the ETS; while our study demonstrates citric acid cycle dependent respiration. Taken together with our study, these results illustrate how different substrates impact BAT mitochondrial respiration; our study using succinate as a fuel focused on ETS function downstream of Complex I (Complex II and beyond) while the *McFarlane et al* (2017) study lends insight on fatty acid supported respiration of the entire ETS and citric acid cycle in addition to fatty acid transport proteins, which is more applicable to studies specifically focusing only on BAT mitochondrial respiration rates.

Perspectives and significance

The mechanisms that facilitate or constrain mitochondrial function across a range of temperatures over the circannual cycle is not fully understood. To examine what impact temperature has on mitochondrial respiration rates from two different tissues, non-thermogenic liver and thermogenic BAT mitochondria were isolated from thirteen-lined ground squirrels across their circannual cycle. Rates of *state 3* and *state 4* respiration, respectively, were measured *in vitro* at 5, 13, 21, 29, and 37ºC fueled by succinate. Results show that liver mitochondria are more sensitive to both temperature and season compared to BAT. In fact, BAT mitochondrial respiration was significantly different

between summer and torpor at 5°C only with torpor having higher respiration rates than summer (Figure 3.1A). Furthermore, torpor mitochondrial respiration rates were higher than all other seasons at the lowest three temperatures measured (5, 13, and 21°C), but not statistically significantly, aside from the previously mentioned. The same trend was not observed in liver mitochondria. Liver mitochondria isolated from squirrels in torpor had respiration rates that were lower at all temperatures compared to the other seasons (Figure 3.1B), although not always significantly (Table 3.1). These results suggest there could be some mechanism in place that suppresses liver mitochondrial respiration during hibernation that is not present during other seasons; perhaps post-translational modifications (Mathers et al, 2016). The opposite could also be true; there could be some mechanism that facilitates maintenance of BAT mitochondrial function across the circannual cycle which is largely temperature insensitive.

Future studies should look at other, more common fatty acids such as palmitic acid and make more comparisons across the circannual cycle, since our study shows that there are differences in BAT mitochondrial respiration when different fuels are used and when mitochondria are isolated at other times of the year, i.e. summer and torpor. To further understand and compare differences between thermogenic and non-thermogenic tissues, liver transcriptome, proteome, and mitoproteome studies could lend insight. These have been obtained for BAT (Ballinger et al, 2015; Hampton et al, 2013), but unlike BAT, liver shows significant suppression across the circannual cycle and is dependent on temperature. Comparing "omic" results from such studies might reveal differences in mRNA and proteins that could account for some of the differences

observed seasonally and at varying temperatures. These studies would not be able to demonstrate post-translational modifications or enzyme modifications that might occur and impact respiration rates so more studies targeting these changes could further our understanding of mitochondrial specific changes that result in functional changes.

CHAPTER 4: Evaluation of Adenosine Production and Transport in a Mammalian Hibernator

SECTION 4.1: RESULTS

To assess protein abundance of the enzymes and transporters involved with adenosine production and transport in thirteen-lined ground squirrel skeletal muscle and BAT, abundance of five proteins were determined via Western blotting. Protein was isolated from skeletal muscle and BAT from four time points throughout the circannual cycle: spring (SP), fall (FALL), torpor (TOR), and interbout arousal (IBA). Western blots using five distinct antibodies identified two differentially expressed proteins, one in skeletal muscle (AMPD1, Figure 4.3) and one in BAT (ADORA1, Figure 4.5), both of which were shown to have differentially expressed mRNA levels (Vermillion et al, 2015; Anderson et al, 2016; Hampton et al, 2013). A third protein, SLC29A1, had differentially expressed mRNA levels (Figure 4.4), but no significant differences in protein expression levels (Figure 4.4).

Protein abundance

Protein expression across the four time points were determined via Western blot and statistically analyzed by one-way ANOVA followed by Tukey's HSD test to identify which groups (or seasons) were different from others (Section 2.8). Proteins expression was considered statistically different when $P < 0.05$. Band density was determined using LI-COR Odyssey® FC (Section 2.4). To normalize the density of each band, protein expression levels were compared to a pooled sample containing one sample from each time point that had approximately the mean protein abundance determined by BCA protein assay (Section 2.4).

All five of the proteins examined are suggested to be influenced by adenosine and/or adenine nucleotides or have a role in the production and transport of adenosine, directly or indirectly (Anderson et al, 2016; Beal et al, 2004; Hampton et al, 2013; Hardie et al, 2012; Plaideau et al, 2014; Zhang et al, 2006). One of the proteins, enzyme AMP-activated protein kinase (AMPK), becomes activated by phosphorylation (AMPK-phosphorylated or AMPK-P*)* in response to increasing AMP: ATP or ADP: ATP ratio (Hardie et al, 2012). In skeletal muscle, *AMPK* (not specified as activated/phosphorylated AMPK or not) mRNA is not differentially expressed, but there is a trend toward higher mRNA expression levels during more active periods such as April, fall, and IBA (Figure 4.1A, Vermillion et al, 2015). There were no significant differences in protein expression levels for AMPK, but there were generally higher expression levels during more active periods (Figure 4.1C), comparable to mRNA levels (Figure 4.1A, Vermillion et al, 2015). Activated AMPK (AMPK-phosphorylated, Phospho T183 alpha 1, Phospho T172 alpha 2) also had no significant differences, but had the highest expression level during the fall (Figure 4.2B).

One way in which AMP can be metabolized in skeletal muscle is by AMP deaminase 1 (AMPD1) to IMP. Recently published skeletal muscle proteome data showed AMPD1 levels were significantly higher in October than torpor; and April was higher than IBA (Anderson et al, 2016). During hibernation (torpor and IBA), there were no significant differences in AMPD1 protein levels (Anderson et al, 2016). Based on Western blot results, levels of AMPD1 were significantly higher in spring than torpor and

IBA (P = 0.003, Figure 4.3C). There were no significant differences between spring and fall, fall and torpor, fall and IBA, and torpor and IBA (Figure 4.3C).

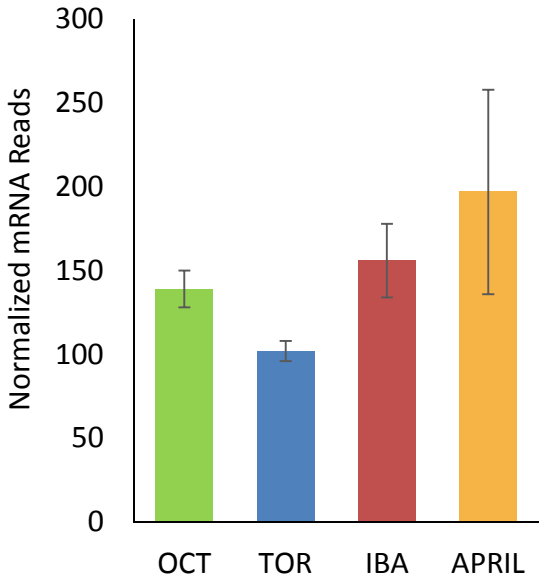
If AMP levels are high and AMP is not being metabolized via AMPD1, AMP can be converted to adenosine (Plaideau et al, 2014) and transported out of skeletal muscle into circulation via equilibrative nucleoside transporter 1/solute carrier family 29 member 1, (SLC29A1; Beal et al, 2004). In skeletal muscle, *SLC29A1* mRNA is significantly lower in April compared to fall, torpor, and IBA (Figure 4.4A; Vermillion et al, 2015). No significant differences in protein levels were identified with Western blotting (Figure 4.4C). However, SLC29A1 protein levels were highest when squirrels were at normothermic temperatures: spring, fall, and IBA (Figure 4.4C).

Adenosine in circulation can bind to the adenosine receptor on BAT, Adenosine A_1 Receptor (ADORA1; Hampton et al, 2013). Levels of *ADORA1* mRNA in BAT are significantly higher in torpor and IBA than they are in April, while October is not significantly different from any season measured (Hampton et al, 2013; Figure 4.5A). However, ADORA1 protein levels are significantly higher during fall than spring and IBA (P< 0.001; Figure 4.5C). Torpor, IBA, and spring were not significantly different, but IBA and spring had the lowest protein expression levels of ADORA1 (Figure 4.5C).

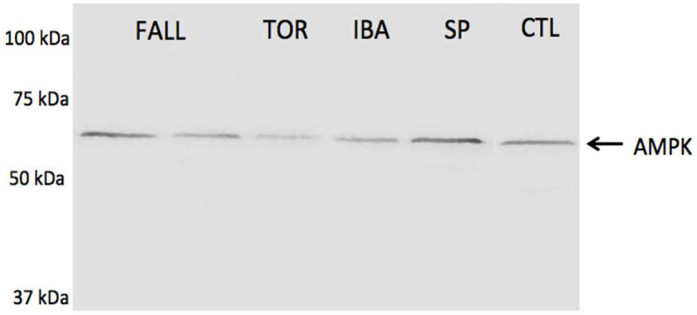
Section 4.1: Tables and Figures

Figure 4.1. Seasonal AMPK levels in skeletal muscle. A) AMPK mRNA expression levels (Vermillion et al, 2015), B) representative AMPK Western blot, and C) densitometric analysis of AMPK Western blots (n=9). 40 µg of skeletal muscle (quadriceps) protein homogenate was loaded in each lane. LI-COR Odyssey® FC was used to measure the density of the ~65 kDa band from Western blots using anti-AMPK antibody. Bands from each of the blots were normalized to a common lane (CTL) with 40 µg pooled skeletal muscle homogenate comprised of one sample from each time point (fall hyperphagic, fall hypophagic, torpor, IBA, and spring). FALL bands on Western blot represent hyperphagic and hypophagic time points, respectively, and were grouped into one FALL time point for analysis. No significant differences were identified in either mRNA or protein expression levels of AMPK. Error bars represent ± standard error. Abbreviations: TOR, torpor; IBA, interbout arousal; SP, spring; CTL, control; AMPK, AMP-activated protein kinase. For densitometric analysis, FALL n = 18, TOR n =9, IBA n = 9, SP n =9.

A.



B.



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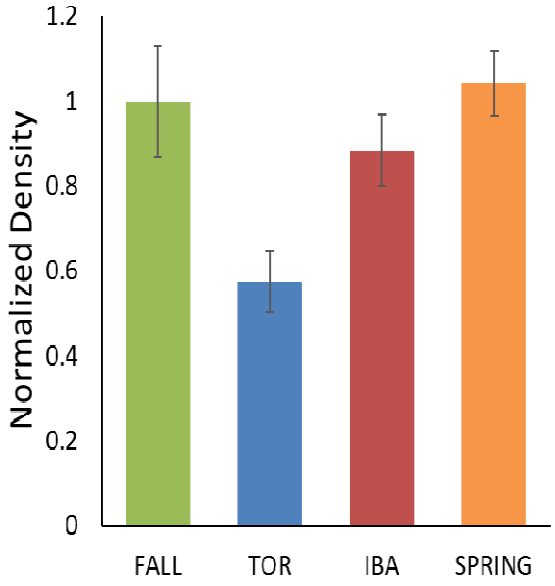
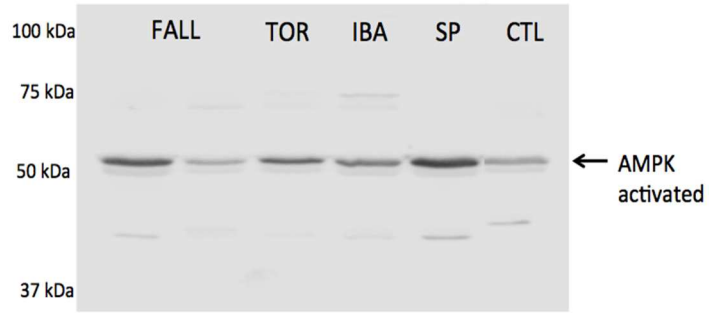


Figure 4.2. Seasonal AMPK activated (Phospho T183 alpha 1, Phospho T172 alpha 2) levels in skeletal muscle. A) representative AMPK activated Western blot and B) densitometric analysis of AMPK activated Western blots (n=9). 40 µg of skeletal muscle (quadriceps) protein homogenate was loaded in each lane. LI-COR Odyssey® FC was used to measure the density of the ~64 kDa band from Western blots using anti- AMPK activated antibody. Bands from each of the blots were normalized to a common lane (CTL) with 40 µg pooled skeletal muscle homogenate comprised of one sample from each time point (fall hyperphagic, fall hypophagic, torpor, IBA, and spring). FALL bands on Western blot represent hyperphagic and hypophagic time points, respectively, and were grouped into one FALL time point for analysis. No significant differences were identified in protein expression levels of activated AMPK. Error bars represent ± standard error. Abbreviations: TOR, torpor; IBA, interbout arousal; SP, spring; CTL, control; AMPK, AMP-activated protein kinase. For densitometric analysis, FALL n = 18, TOR n =9, IBA n = 9, SP n =9.

A.



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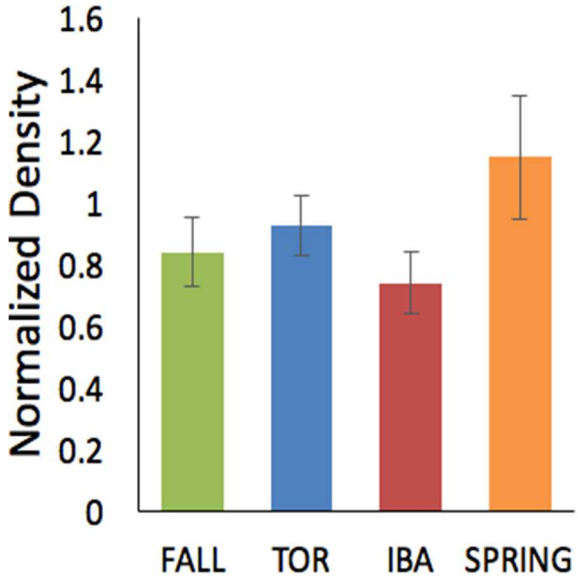
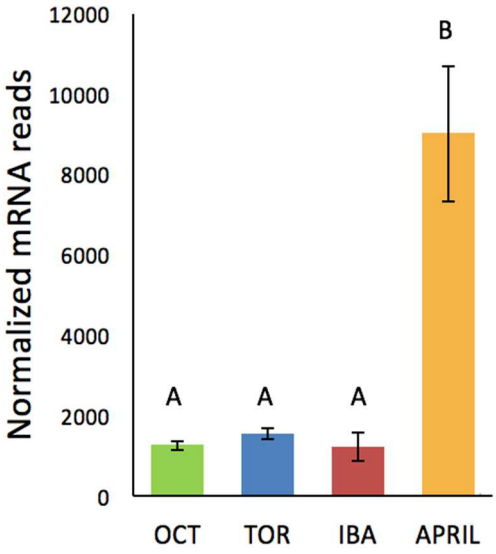
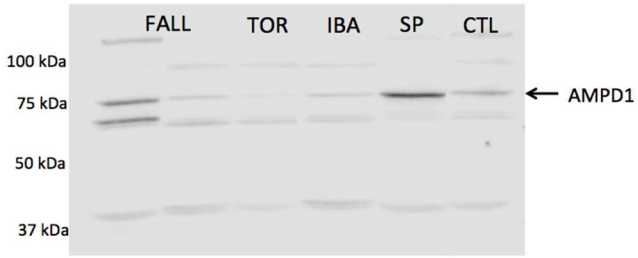


Figure 4.3. Seasonal AMPD1 levels in skeletal muscle. A) AMPD1 mRNA expression levels (Vermillion et al, 2015), B) representative AMPD1 Western blot, and C) densitometric analysis of AMPD1 Western blots (n=9). Time points that do not share the same letter are significantly different (Tukey's highly significant difference test). 40 µg of skeletal muscle (quadriceps) protein homogenate was loaded in each lane. LI-COR Odyssey® FC was used to measure the density of the ~87 kDa band from Western blots using anti-AMPD1 antibody. Bands from each of the blots were normalized to a common lane (CTL) with 40 µg pooled skeletal muscle homogenate comprised of one sample from each time point (fall hyperphagic, fall hypophagic, torpor, IBA, and spring). FALL bands on Western blot represent hyperphagic and hypophagic time points, respectively, and were grouped into one FALL time point for analysis. mRNA expression levels of AMPD1 are significantly greater during SPRING than OCT, TOR, and IBA. Protein levels of AMPD1 are significantly higher during SPRING compared to TOR and IBA. FALL AMPD1 protein expression is not significantly different than the other three time points. Error bars represent ± standard error. Abbreviations: TOR, torpor; IBA, interbout arousal; SP, spring; CTL, control; AMPD1, AMP Deaminase 1. For densitometric analysis, FALL n = 18, TOR n =9, IBA n = 9, SP n =9.

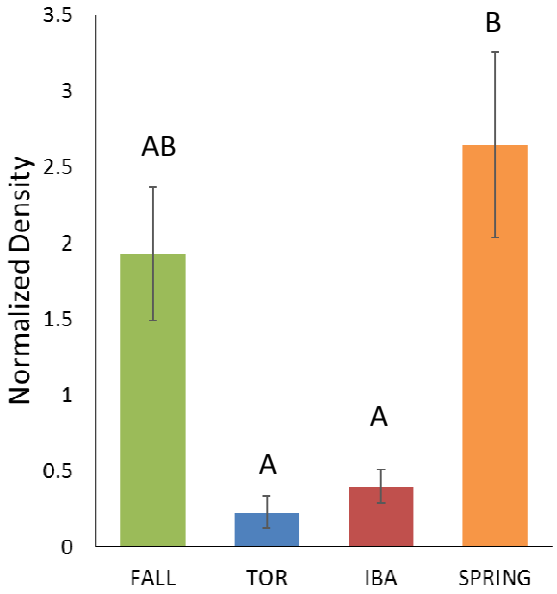
A.



B.



C.


3.5
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Normalized Density
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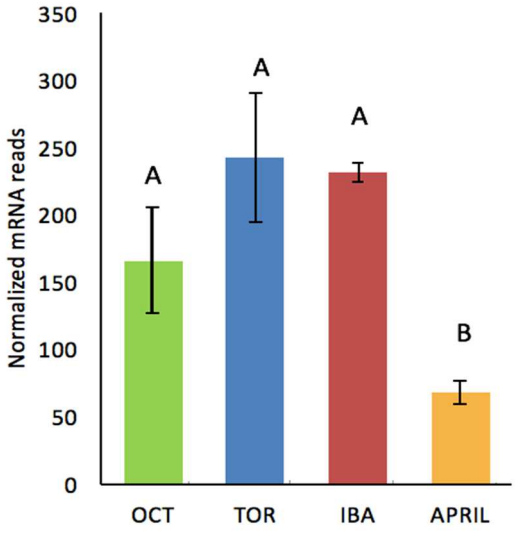
IBA
SPRING

Figure 4.4. Seasonal SLC29A1 levels in skeletal muscle. A) SLC29A1 mRNA expression levels (Vermillion et al, 2015), B) representative SLC29A1 Western blot, and C) densitometric analysis of SLC29A1 Western blots (n=9). Time points that do not share the same letter are significantly different (Tukey's highly significant difference test). 40 µg of skeletal muscle (quadriceps) protein homogenate was loaded in each lane. LI-COR Odyssey® FC was used to measure the density of the ~50 kDa band from Western blots using anti-SLC29A1 antibody. Bands from each of the blots were normalized to a common lane (CTL) with 40 µg pooled skeletal muscle homogenate comprised of one sample from each time point (fall hyperphagic, fall hypophagic, torpor, IBA, and spring). FALL bands on Western blot represent hyperphagic and hypophagic time points, respectively, and were grouped into one FALL time point for analysis. mRNA expression levels of SLC29A1 are significantly lower during SPRING than OCT, TOR, and IBA. No significant differences in SLC29A1 protein expression levels were identified. Error bars represent ± standard error. Abbreviations: TOR, torpor; IBA, interbout arousal; SP, spring; CTL, control; SLC29A1, Solute Carrier Family 29 Member 1. For densitometric analysis, FALL n = 16, TOR n =8, IBA n = 9, SP n =9.

A.



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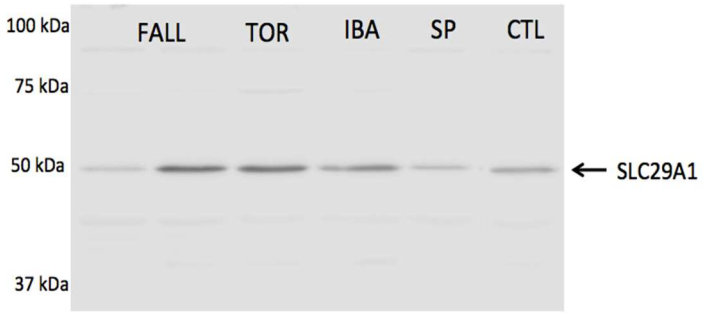


C.

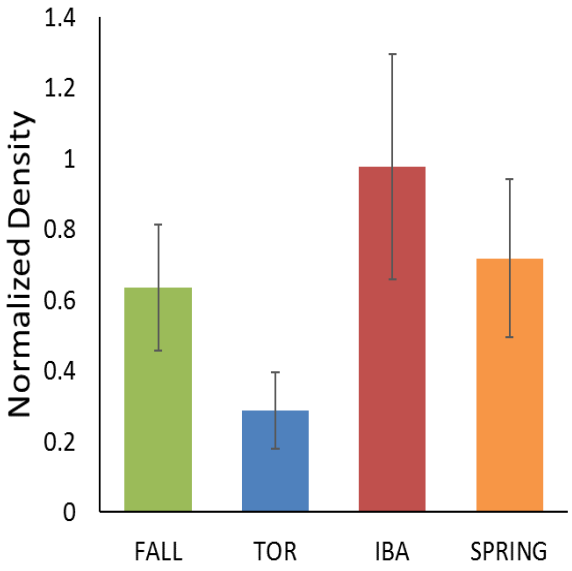
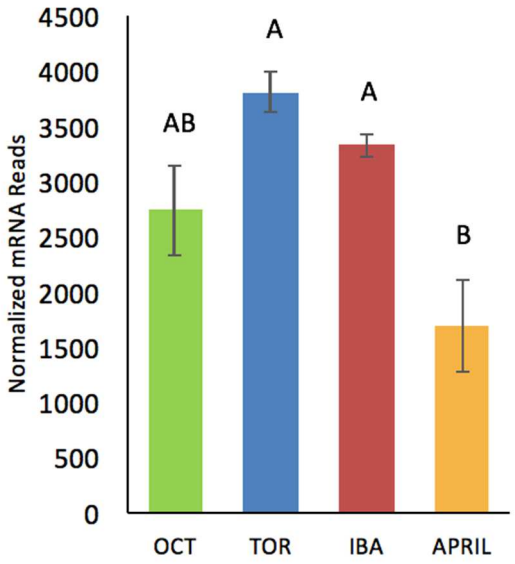
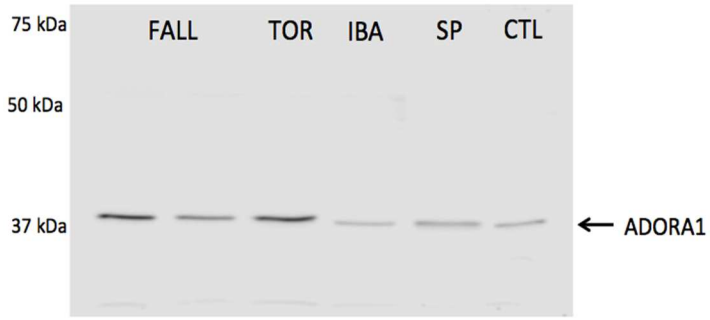


Figure 4.5. Seasonal ADORA1 levels in brown adipose tissue (BAT). A) ADORA1 mRNA expression levels (Hampton et al, 2013), B) representative ADORA1 Western blot, and (C) densitometric analysis of ADORA1 Western blots (n=9). Time points that do not share the same letter are significantly different (Tukey's highly significant difference test). 40 µg of BAT protein homogenate was loaded in each lane. LI-COR Odyssey® FC was used to measure the density of the ~37 kDa band from Western blots using anti-ADORA1 antibody. Bands from each of the blots were normalized to a common lane (CTL) with 40 µg pooled BAT homogenate comprised of one sample from each time point (fall hyperphagic, fall hypophagic, torpor, IBA, and spring). FALL bands on Western blot represent hyperphagic and hypophagic time points, respectively, and were grouped into one FALL time point for analysis. mRNA expression levels of ADORA1 are significantly lower during SPRING than TOR and IBA. OCT ADORA1 mRNA levels are not significantly different than SP, TOR, and IBA. Protein expression of ADORA1 is significantly higher during the FALL compared to SPRING and IBA. TOR ADORA1 protein expression levels are not significantly different than the other three time points sampled. Error bars represent ± standard error. Abbreviations: TOR, torpor; IBA, interbout arousal; SP, spring; CTL, control; ADORA1, adenosine A1 receptor. For densitometric analysis, FALL n = 18, TOR n =9, IBA n = 9, SP n =9.

A.



B.


75 kDa
50 kDa
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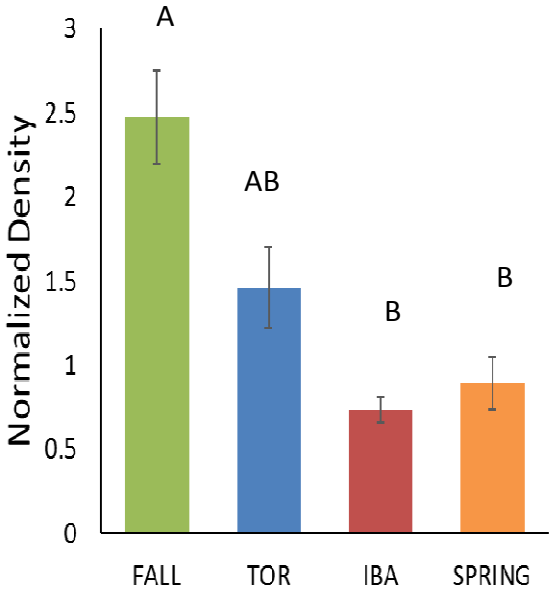
C.


Normalized Density
3
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SECTION 4.2: DISCUSSION

How the expression levels of specific proteins change the metabolism and transport of certain chemical messengers and the receptors that transmit the signals across the circannual cycle of thirteen-lined ground squirrels is not fully known. One such chemical is adenosine. Adenosine is known to be important for sleep, energy homeostasis, and hibernation induction (Drew and Jinka, 2012; Hampton et al, 2013; Swoap et al, 2007; Zhang et al, 2006) therefore understanding how it could be regulated seasonally is important.

To examine what impact season has on several proteins involved in adenosine production and transport in the two tissues most responsible for thermogenesis, skeletal muscle and BAT, protein expression level was measured by Western blot at four time points across the circannual cycle; fall, torpor, IBA, and spring. We anticipated season to have a significant influence on protein levels, based on mRNA expression. To test this hypothesis, we measured the levels of five proteins involved in adenosine and/or adenine nucleotides metabolism or transport (Anderson et al, 2016; Beal et al, 2004; Hampton et al, 2013; Hardie et al, 2012; Plaideau et al, 2014; Zhang et al, 2006). Although mRNA expression data is available for all proteins (Hampton et al, 2013; Vermillion et al, 2015; Anderson et al, 2016), it does not always correlate to protein levels (Foss et al, 2011).

Overall, only AMPD1 (Figure 4.3) in skeletal muscle and ADORA1 (Figure 4.5) in BAT had differentially expressed protein levels. However, AMPK protein expression followed the same trend as mRNA (Figures 4.1 and 4.2). Although SLC29A1 protein expression levels were not significantly different, the mRNA levels were and more

surprisingly, perhaps, the protein and mRNA levels appeared to have opposite expression (Figure 4.4). This was also observed for ADORA1 (Figure 4.5). Although less significant differences were found than predicted by mRNA data, these results suggest there are seasonal differences in protein expression level and that could influence production and transportation of adenosine as well as the effect adenosine has on target tissues throughout the circannual cycle.

AMPK proteins levels are relatively constant throughout the circannual cycle

Enzyme AMP-activated protein kinase (AMPK), becomes activated by phosphorylation in response to increasing AMP: ATP and ADP: ATP ratios (Hardie et al, 2012). Its downstream effects aim to increase ATP production and decrease ATP utilization (Hardie et al, 2012) at both the cellular and whole organismal level (Drew and Jinka, 2012). Activated AMPK stimulates catabolic pathways while also inhibiting anabolic pathways. Increasing ATP levels inhibit additional activation of AMPK which provides a mechanism for rapid changes in response to cellular energy levels (Drew and Jinka, 2012). In skeletal muscle, neither *AMPK* mRNA nor AMPK protein levels were differentially expressed in the ground squirrel, but typically had higher mRNA expression levels during more active periods such as April, October, and IBA (Figure 4.1A and C; Vermillion et al, 2015), providing the ability for rapid response to changes in energy status. Activated AMPK (Phospho T183 alpha 1, Phospho T172 alpha 2) also showed no significant differences, but had the highest expression level during the fall (Figure 4.2B). Since a substantial amount of ATP would be used in skeletal muscle during periods of

high activity, throughout spring to fall and during arousal from hibernation, it makes sense that AMPK would be abundant in this tissue.

Initial rewarming from torpor is almost entirely dependent on BAT function, but when body temperature reaches ~ 16ºC, shivering thermogenesis in skeletal muscle also contributes (Cannon and Nedergaard, 2004). Shivering thermogenesis would consume a large amount of ATP resulting in an increase in AMP potentially promoting an increase in AMPK activation. When activated, AMPK stimulates fatty acid oxidation and mitochondrial biogenesis while inhibiting fatty acid and protein synthesis, among other pathways (Drew and Jinka, 2012). Because fatty acids are necessary for BAT non-shivering thermogenesis (Cannon and Nedergaard, 2004) and activation of AMPK stimulates the oxidation of fatty acids, AMPK activation could be an important mediator during the torpor – arousal cycles of hibernation.

Low AMPD1 expression levels during hibernation could result in an accumulation of AMP

In skeletal muscle, AMPK and AMPD1 could have opposing activity levels throughout the year, which could serve as an influential mechanism for the metabolic reduction seen in hibernation. In other ground squirrel studies looking at protein levels and activity of AMPK and AMP deaminase 2 (AMPD2, liver specific isoform of AMPD) in the liver, researchers saw opposing enzyme activity; when AMPD2 was most active outside of hibernation, AMPK activity was lower and vice versa (Lanaspa et al, 2015). AMP deaminse catalyzes a rate-limiting step that converts AMP to inosine

monophosphate (IMP) and creates ammonia (NH_3) as a byproduct (Plaideau et al, 2014). Therefore, AMPD could serve as a regulator of the AMP: ATP ratio.

The activity of AMPK could be decreased during spring, summer, and fall when AMPD is more active because of increased AMP breakdown via AMPD and therefore reduced AMP available to active AMPK. Furthermore, in the liver, AMPD2 metabolism byproduct uric acid has been found to be an inhibitor of AMPK (Lanaspa et al, 2012). If uric acid levels are high, this could trigger fatty acid synthesis in the liver during periods of high AMPD2 activity (Lanaspa et al, 2015). During the summer when AMPD2 protein levels were elevated, researchers found an increase in uric acid and IMP, the end-product AMP metabolism by AMPD2, in addition to an increase in enzymes associated with fat synthesis (Lanaspa et al, 2015). Simultaneously, activated AMPK levels were low as well as the potential for fatty acid oxidation (Lanaspa et al, 2015).

If the opposing activity seen in the liver between AMPK and AMPD2 is similar in skeletal muscle, with AMPK and AMPD1 (skeletal muscle specific isoform of AMPD), this would facilitate fatty acid oxidation during hibernation when AMPD1 activity is low and AMPK activity is increased thereby providing fuel for mitochondria. Recently published proteome data showed AMPD1 levels were significantly higher in October than torpor; and April was higher than IBA (Anderson et al, 2016). During hibernation, there were no significant differences between torpor and IBA AMPD1 protein levels (Anderson et al, 2016). Based on Western blot results, levels of AMPD1 were significantly higher in spring than torpor and IBA (Figure 4.3C). There were no significant differences between spring and fall, fall and torpor, fall and IBA, and torpor

and IBA (Figure 4.3C). Variances in expression levels between Western blot and proteome could be due to timing differences of animal sacrifice.

If increased protein levels of AMPD1 directly correlate to increased AMPD1 activity, AMPD1 could be more active during spring, summer, and fall while AMPK activity is lower and/or reduced by uric acid. This could promote fatty acid synthesis and serve as a mechanism to increase fat storage in skeletal muscle that could be used throughout hibernation. Low serum inosine levels during late torpor, arousal, and IBA (Epperson et al, 2011) could provide further support for low AMPD1 activity levels during hibernation since AMPD1 metabolizes the conversion of AMP to IMP which can be further broken down to inosine via 5'-nucleotidases. Inosine can be transported out of cells primarily by equilibrative nucleoside transporter 2/solute carrier family 29 member 2 (SLC29A2) which has high mRNA expression levels in April compared to October, torpor, and IBA (Vermillion et al, 2015) coupled with an increase in serum inosine levels during spring and summer (Epperson et al, 2011).

Enhanced adenosine transport out of skeletal muscle cells via SLC29A1 at normothermic body temperatures

The significant decrease in AMPD1 levels, and possible enzyme activity, could result in high AMP levels as it is not being metabolized to IMP, via AMPD1. Instead, AMP can be converted to adenosine (Plaideau et al, 2014) and transported out of skeletal muscle cells and into circulation via the equilibrative nucleoside transporter 1/solute carrier family 29 member 1, SLC29A1 (Beal et al, 2004). Adenosine and adenine

nucleotides are transported across the cell membrane in either direction, depending on the surrounding concentrations, by SLC29A1, which has a higher affinity for adenosine. In skeletal muscle, *SLC29A1* mRNA is significantly lower in April compared to October, torpor, and IBA (Figure 4.4A, Vermillion et al, 2015). No significant differences in protein levels were identified with Western blotting (Figure 4.4C). However, SLC29A1 protein levels were highest when squirrels were at normothermic temperatures: spring, fall, and IBA (Figure 4.4C).

Adenosine and AMP could accumulate in the skeletal muscle as shivering thermogenesis occurs during arousal, due to high utilization of ATP and reduced ability for metabolism of AMP to IMP. Instead, accumulating AMP could be converted to adenosine by alternative pathways (Plaideau et al, 2014). Adenosine in the serum is higher during IBA than late torpor and arousal when body temperature is between 7 and 12^{o}C (Epperson et al, 2011), before shivering thermogenesis begins. The increase in adenosine levels during IBA could be due to high levels of SLC29A1 in skeletal muscle facilitating transport of adenosine that accumulated during arousal to serum. During IBA, it has been suggested that squirrels sleep (Daan et al, 1991) and adenosine is known to promote sleep (Drew and Jinka, 2012); therefore, increased serum adenosine levels could be achieved through increased transport from skeletal muscle via SLC29A1 and promote sleep during IBA. However, the difference between sleep and hibernation could be realized by a seasonal change in sensitivity of BAT adenosine receptor ADORA1 (Drew and Jinka, 2012) that may or may not occur between torpor and IBA.

Pre-hibernation contains significantly higher levels of ADORA1 protein

Adenosine in circulation can bind to the adenosine receptor, ADORA1, on BAT cell membrane and serve as a potential mechanism to inhibit heat production via mitochondrial UCP1 through blockage of free fatty acid release and a cascade of events (Gnad et al, 2014; Hampton et al, 2013). In WAT, interaction between ADORA1 and adenosine causes inhibition of lipolysis and prevents the release of free fatty acids that are needed for activation of BAT non-shivering thermogenesis via UCP1 (Gnad et al, 2014). Levels of *ADORA1* mRNA in BAT are significantly higher in torpor and IBA than they are in April, while October is not significantly different from any season measured (Hampton et al, 2013; Figure 4.5A). However, ADORA1 protein levels were significantly higher during fall than spring and IBA while torpor had intermediate, non-significantly different, levels of ADORA1 (Figure 4.5C).

ADORA1 levels in the fall could be increased in preparation for hibernation since transcription and translation levels are greatly depressed below 18^{o}C body temperature (van Breukelen and Martin, 2001) and when coupled with potentially low levels of ATP throughout hibernation protein synthesis could be inhibited (Drew and Jinka, 2012). Furthermore, the quantity of ADORA1 may not play a significant role outside the hibernation season if the activation with adenosine only induces a response during winter (Jinka et al, 2011). Changes in ADORA1 protein levels alone might not influence the response to adenosine, or adenosine agonists so the increase we see in protein levels during the fall could be in preparation for decreased rates of protein synthesis.

If ADORA1 does show seasonal sensitivity to adenosine, increased serum adenosine levels in July and August (Epperson et al, 2011) might not have much of an

influence in controlling BAT thermogenesis and instead, the inhibition could be controlled entirely by other thermoregulatory pathways, such as signaling from cutaneous tissue to the brain (Nakamura and Morrison, 2007 and 2008). Additionally, in hamster and rat studies, adenosine has been suggested to reduce BAT sensitivity to catecholamines, such as norepinephrine which is known to stimulate BAT non-shivering thermogenesis, perhaps providing more control over heat production (Cannon and Nedergaard, 2004; Gnad et al, 2014) throughout the circannual cycle.

Perspectives and Significance

Skeletal muscle accounts for approximately 40% of an organisms' body weight and accounts for 20-30% of basal metabolic rate in non-obese humans (Zurlo et al, 1990). Skeletal muscle could also contribute to whole organismal rewarming via shivering thermogenesis (Cannon and Nedergaard, 2004). Furthermore, skeletal muscle can change the concentration of chemical messengers in the blood by serving as an endocrine-like organ (Pratesi et al, 2013). Skeletal muscle could transport chemicals into muscle cells when the serum concentration is saturating, or release chemicals when serum concentrations are low, such as adenosine (Richter et al, 1998).

The present study examined the influence of season on five proteins involved in adenine nucleotide (AMP, ADP, and ATP) metabolism, production, and transport in thirteen-lined ground squirrels. Adenosine and adenine nucleotides have been implicated in sleep, energy homeostasis, and hibernation induction through receptor dependent,

biochemical, and bioenergetic processes (Drew and Jinka, 2012; Hampton et al, 2013; Swoap et al, 2007; Zhang et al, 2006). One likely target of adenosine is BAT.

Adenosine binding to the adenosine receptor, ADORA1, could result in inhibition of adenylate cyclase activity and the downstream process of heat production via non-shivering thermogenesis (Hampton et al, 2013). Brown adipose tissue only makes up around 5% of the body mass in small rodents, such as ground squirrels, yet it can increase the entire body's respiration up to 10-fold (Nicholls and Ferguson, 2013). Brown adipose tissue is responsible for initiating and facilitating whole organism rewarming from torpor to IBA, a body temperature shift of approximately 32ºC, within a couple of hours (Cannon and Nedergaard, 2004; Schwartz et al, 2015a; Schwartz et al, 2015b). Controlling heat production could be crucial for hibernators as they cycle between torpor and IBA, and maintain a normothermic body temperature during active seasons. Therefore, examining protein levels involved in adenosine production and transport across the circannual cycle of thirteen-lined ground squirrels could explain how heat production is controlled body-wide by regulation of the two primary tissues responsible for heat production through shivering and non-shivering thermogenesis.

Future studies should measure the activity of AMPK and AMPD1 in skeletal muscle across the circannual cycle since other studies show there are differences in the liver (Lanaspa et al, 2015) that could have substantial impacts for hibernators especially in a tissue as important as skeletal muscle. Additionally, looking at the enzyme activity of skeletal muscle enzymes (AMPK and AMPD1) and transporter (SLC29A1) at different temperatures could lend insight to how well they function at the low

temperatures reached during torpor. More specifically for AMPD1, decreased protein levels and activity could have an impact on the production of nitrogenous waste since little ammonia would be produced from the metabolism of AMP. This could be especially vital throughout the hibernation season when hibernators do not consume any food and rarely, if at all, drink and urinate (Anderson et al, 2016). However, during arousal to IBA, squirrels rely on their skeletal muscle to rewarm so the amount of urine produced could be lowered by controlling the amount of nitrogenous waste produced (Anderson et al, 2016). Lastly, since adenosine has been implicated in inhibition of BAT non-shivering thermogenesis and there is differential expression of skeletal muscle proteins involved in the production and transport, coupled with changes in serum levels throughout the circannual cycle (Epperson et al, 2011), it would be extremely beneficial to determine skeletal muscle concentrations of adenosine and adenine nucleotides, especially during the torpor – arousal – return to torpor cycle at various body temperatures both before, during, and after shivering and non-shivering thermogenesis.

CHAPTER 5: Brown Adipocyte Respiration in the Presence of Adenosine

SECTION 5.1: RESULTS

To assess the impact adenosine has on the function of brown adipocytes in thirteen-lined ground squirrels, isolated adipocyte respiration rates were measured in the presence of adenosine after stimulation with non-specific β-adrenoreceptor agonist isoproterenol. Furthermore, to evaluate temperature and seasonal influence, respiration rates were measured at two temperatures (10 and 37^{o}C) from squirrels sacrificed at four time points; spring (SP), fall (FALL), torpor (TOR), and interbout arousal (IBA). We hypothesized that respiration rates in the presence of adenosine would not be significantly different than basal rates because adenosine is thought to serve as a potential mechanism to inhibit non-shivering thermogenesis heat production via BAT mitochondria through blockage of free fatty acid release and a cascade of events (Gnad et al, 2014; Hampton et al, 2013). With seasonal changes in serum adenosine levels (Epperson et al, 2011) and the potential seasonal change in BAT adenosine A_1 receptor (ADORA1) sensitivity to adenosine (Jinka et al, 2011) coupled with significant seasonal changes in ADORA1 protein levels (Chapter 4), we hypothesized that season would have an impact on brown adipocyte respiration rates in the presence of adenosine.

Brown adipocyte respiration rates at each temperature and across the four time points were determined via Clark-type oxygen electrode from Hansatech Instruments. Adipocyte respiration rates were statistically analyzed by split-plot ANOVA followed by Tukey's HSD test to identify significant differences between season and at each temperature tested (Section 2.8). Respiration rates were considered statistically different when $P < 0.05$. Respiration rates were measured after the adipocytes were incubated for

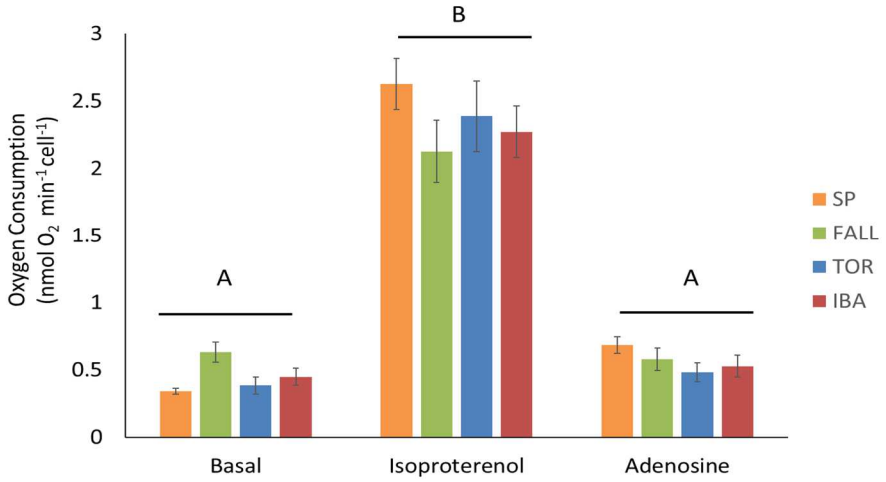
three minutes with no additional substrate, for the basal rate, or following substrate addition, for isoproterenol and adenosine.

After significantly stimulating brown adipocyte respiration with isoproterenol, for three minutes, addition of adenosine resulted in the return of respiration to basal levels at both temperatures (Figure 5.1 A and B). However, there was no change in this response between seasons (Figure 5.1 A and B). Additional comparisons were performed to understand whether decreases in metabolism are due to passive thermal effects or whether metabolism is actively inhibited using a Q_{10} analysis. Temperature coefficients (Q_{10}) reflect the capacity of organisms to change their metabolic rate relative to changes in temperature using the equation $Q_{10} = (R_2 / R_1) \wedge (10 / (T_2 - T_1))$, where the low temperature (10^oC) was set at T_1 and compared to 37^oC, set at T_2 (Baust and Baust, 2007; Geiser, 1988; Staples, 2014). The second rate measurement (R_2) corresponded to the average respiration rate at 37^oC and the respiration rate corresponding to the 10^oC temperature set at R_1. Based on these comparisons, all but one Q_{10} values fall between 1 and 2 (Table 5.1) suggesting temperature insensitivity (Guppy and Withers, 1999; Snapp and Heller, 1981; Staples and Brown, 2008). Spring basal respiration had the only Q_{10} value higher than 2 at 2.27 (Table 5.1).

Section 5.1: Tables and Figures

Figure 5.1. Isolated brown adipocyte basal, isoproterenol stimulated, and adenosine present respiration rates at A) 10ºC and B) 37ºC. Respiration rates were measured after the adipocytes were incubated for three minutes with no additional substrate, for the basal rate, or following substrate addition, for isoproterenol and adenosine. Significant differences between basal, isoproterenol, and adenosine respiration rates at any given temperature and season determined by Tukey's highly significant difference test. Significant differences are indicated between the treatments by the differences in the letters above. Data are expressed as means ± SE; FALL n = 14, TOR n = 10, IBA n = 10, SP n = 10. Abbreviations: SP, spring; TOR, torpor; IBA, interbout arousal

A.



B.

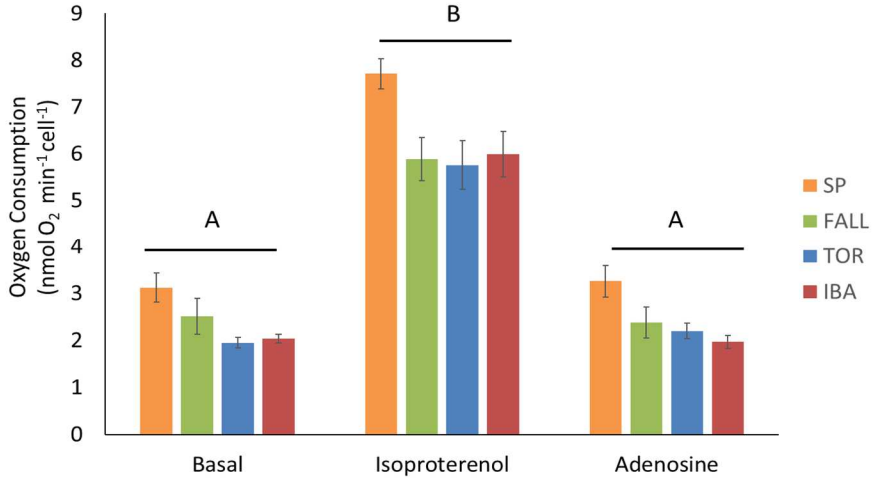


Table 5.1. Mean respiration rate represented as a Q_{10} comparison for isolated brown adipocytes across the circannual cycle for a temperature range of 37°C to 10°C. Temperature coefficient (Q_{10}) values reflect the capacity of organisms to change their metabolic rate relative to changes in temperature (Foss et al, 2011 and Staples, 2014). The low temperature (10°C) was set at T_1 and compared to 37°C, set at T_2. The second rate measurement (R_2) corresponded to the average respiration rate at 37°C and the 10°C rate set at R_1. The Q_{10} equation is: $Q_{10} = (R_2 / R_1) \wedge (10 / (T_2 - T_1))$ (Baust and Baust, 2007). Abbreviations: TOR, torpor; IBA, interbout arousal

Brown adipocytes	SPRING	FALL	TOR	IBA
BASAL	2.27	1.66	1.82	1.75
ISOPROTERENOL	1.49	1.46	1.38	1.43
ADENOSINE	1.78	1.69	1.76	1.63

SECTION 5.2: DISCUSSION

The thirteen-lined ground squirrel undergoes extreme and rapid shifts in body temperature during hibernation as it cycles between torpor and IBA. This requires regulated changes in the physiology of BAT that is responsible for initiating and facilitating whole-organism rewarming by producing heat via non-shivering thermogenesis. However, once normothermic body temperature is reached during arousal, production of additional heat is not needed; therefore, regulation of this whole-body heating and cooling is important. Since elevated adenosine has been suggested to serve as a potential means to inhibit BAT heat production by receptor-dependent processes, we measured the respiration rates of isolated brown adipocytes in the presence of adenosine after stimulation with isoproterenol using a Clark-type oxygen electrode. To test for any seasonal or temperature impacts, brown adipocytes were isolated from squirrels at four time points across the circannual cycle (fall, torpor, IBA, and spring) and respiration rates were measured at two temperatures (10 and 37^{o}C). Overall, season did not appear to have an influence on respiration rates, but isolated brown adipocytes were sensitive to adenosine addition at both temperatures, as indicated by a return from stimulated respiration rates to basal levels upon adenosine addition (Figure 5.1 A, B).

Additional Q_{10} analysis was conducted to evaluate whether decreases in metabolism were due to passive thermal effects or actively inhibited. Based on Q_{10} comparisons, most brown adipocyte Q_{10} values fall between 1 and 2 (Table 5.1) suggesting isolated brown adipocytes are insensitive to temperature regardless of season and presence of substrate. Spring basal respiration had the only Q_{10} value higher than 2

at 2.27 (Table 5.1) suggesting more temperature sensitivity, but passive thermal effects are sufficient to account for any decreases in respiration rates (Guppy and Withers, 1999; Snapp and Heller, 1981; Staples and Brown, 2008). Season does not appear to have any impact on isolated brown adipocyte respiration rates (Figure 5.1 A, B). However, any potential seasonal changes may have been reversed during the adipocyte isolation process since it requires extended periods of incubation at high temperatures (37^{o}C, McFarlane et al, 2017).

Although no seasonal changes were observed in isolated brown adipocyte sensitivity to adenosine *in vitro*, results from *in vivo* brain studies by *Jinka et al* (2011), suggest otherwise. When treating arctic ground squirrels, also obligate hibernators, with ADORA1 agonist N^6-cyclohexyladenosine (CHA) *in vivo*, administered into the lateral ventricle throughout the circannual cycle, *Jinka et al* (2011) found summer squirrels only experienced moderate decreases in body temperature and oxygen consumption. After the animals had begun preparing for hibernation with spontaneous, shallow torpor bouts, but were not yet in the middle of the hibernation season, CHA administration resulted in mixed outcomes with some animals displaying torpor-like response and others responded the same as summer animals (Jinka et al, 2011). However, when CHA was administered again during an IBA in the middle of the hibernation season, torpor was induced in all squirrels (Jinka et al, 2011). These results suggest the induction and maintenance of torpor is dependent on, and can be explained by, signaling activation in an ADORA1 dependent manner within the central nervous system, but adenosine is most likely not the only neuromodulator necessary to induce torpor (Jinka et al, 2011).

Perhaps not only seasonal changes in the sensitivity of ADORA1 occurs, but also phase of the entire torpor-arousal cycle. Studies in Syrian hamsters found that intracerebroventricular injection of adenosine and adenine nucleotides results in body temperature decreases and a hypothermic effect (Tamura et al, 2005). When ADORA1 antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) was injected intracerebroventricularly during adenosine-induced entrance from euthermia into the maintenance phase of hypothermia resulted in increased body temperature and lessened the hypothermic effects caused by adenosine (Tamura et al, 2005). However, when CPT was injected later during the maintenance phase of hypothermia, there was no impact on body temperature (Tamura et al, 2005). When CHA was administered, a more rapid cooling rate occurred outside of the hibernation season compared to during the hibernation season in Syrian hamsters (Tamura et al, 2005), which is different than what was observed in arctic ground squirrels (Jinka et al, 2011). Therefore, there could be some additional differences in how organisms respond and the pathways involved with hypothermia vs. torpor (Tamura et al, 2005). Nonetheless, these results provide more support that induction and maintenance of torpor or hypothermia is dependent on signaling activation in an ADORA1 dependent manner, but also suggests that adenosine is not involved in maintenance of depressed body temperature (Tamura et al, 2005).

Further support for the induction of a torpor response by adenosine came from mouse studies. In these studies, it has been suggested that AMP induces a hypothermic or torpor-like state when administered peripherally (Zhang et al, 2006). However, when tested again, along with other adenine nucleotides (ADP and ATP), a similar hypothermic

response was observed (Swoap et al, 2007). It has therefore been suggested that it is not adenine nucleotides directly that induce the torpor-like state, but rather adenosine that is produced from nucleotide de-phosphorylation by ecto-5'-nucleotidases (Swoap et al, 2007). Furthermore, blockage of the adenosine receptor in mice prior to AMP injection completely prevented the torpor-like response in mice providing more support that the adenosine effect is realized through a receptor dependent process (Swoap et al, 2007; Iliff and Swoap, 2012).

A similar response with CHA was also seen in rats, a non-hibernating species (Tupone et al, 2013). Rats were held at an ambient temperature of 15^{o}C for six hours and injected with ADORA1 agonist CHA (Tupone et al, 2013). After intracerebroventricular CHA injection, core and BAT temperatures and heart rate declined and were maintained at a steady rate (Tupone et al, 2013). Similar to the decline in heart rate in thirteen-lined ground squirrels entering torpor, the rats experienced frequently skipped heart beats (Tupone et al, 2013). The hypothermic response observed in rats was comparable to the torpor response in obligate hibernators characterized by virtually no motor activity, minimal responsiveness to stimuli, and no eating or drinking with occasional urination (Tupone et al, 2013). Furthermore, after increasing the room temperature from 15 to 28^{o}C, the rats began to rewarm and returned to normothermic rates and behaviors with no obvious signs of stress (Tupone et al, 2013). However, the rewarming response to changing room temperature also suggests the return to a normothermic state was due to skin rewarming rather than declining effectiveness of administered CHA (Tupone et al, 2013). When ADORA1 was blocked with ADORA1 agonist CPT prior to CHA

administration, the inhibition of BAT thermogenesis that occurred with CHA alone was prevented (Tupone et al, 2013). These results further support the idea that ADORA1 activation is necessary to inhibit BAT function.

We see significant seasonal differences in ADORA1 protein expression levels in BAT (Chapter 4) coupled with seasonal changes in serum adenosine levels (Epperson et al, 2011) in thirteen-lined ground squirrels. Brown adipose tissue ADORA1 protein levels are significantly higher during the fall compared to IBA and spring, while torpor has intermediate levels (Chapter 4). High levels of ADORA1 may be especially important during the fall when squirrels are preparing for hibernation by gradually decreasing body temperature which occurs before the decline in food intake (Drew et al, 2016). The increase in ADORA1 protein could also be coupled with an increase in ADORA1 sensitivity, as seen in arctic ground squirrels (Drew et al, 2016). To inhibit non-shivering thermogenesis during torpor, there are higher serum adenosine levels during entrance to torpor and IBA compared to late torpor and arousal (prior to skeletal muscle shivering, Epperson et al, 2011). Both IBAs and the subsequent entrance into torpor is when BAT thermogenesis needs to be inhibited so that animals can become hypothermic.

Perspectives and Significance

Brown adipose tissue accounts for at most 5% of body mass in small mammals such as the thirteen-lined ground squirrel, yet it can facilitate rapid, whole-organismal rewarming via non-shivering thermogenesis (Nicholls and Ferguson, 2013). This

important function occurs frequently as thirteen-lined ground squirrels readily arouse from torpor to IBA throughout the hibernation season. The major thermogenic function of BAT is primarily due to mitochondria containing UCP1 that uncouples the ETS from oxidative phosphorylation to produce heat. This thermogenic capacity and activity of BAT is probably not necessary at normothermic body temperatures; therefore, it is presumed that BAT function changes with body temperature and season.

Although it is known that adenosine reduces lipolysis in isolated white adipocytes (Szillat and Bukowiecki, 1983), it is unknown what impact adenosine has on isolated brown adipocytes from an obligate hibernator. Free fatty acids liberated from lipolysis are used as the primary fuel source throughout hibernation and thus, are crucial for brown adipocyte function as a thermogenic tissue. The present study examined the influence adenosine has on respiration rates from the isolated brown adipocytes of thirteen-lined ground squirrels across the circannual cycle. After stimulating respiration with non-specific β-adrenoreceptor agonist isoproterenol, we predicted that the addition of adenosine would return brown adipocyte respiration rates to basal levels. Furthermore, we examined seasonal and temperature impacts by measuring respiration rates of isolated brown adipocytes from four seasonal time points: fall (FALL), spring (SP), torpor (TOR), and interbout arousal (IBA) at two temperatures (10 and 37^{o}C). Results show that season does not appear to have an influence on respiration rates. However, isolated brown adipocytes were sensitive to adenosine addition at both temperatures, as indicated by a return from stimulated respiration rates to basal levels upon adenosine addition (Figure 5.1 A, B).

Future studies on isolated brown adipocytes should examine the effect of adenine nucleotides on respiration rates. It would also be interesting to measure the rate of brown adipocyte cellular lipolysis with and without adenosine present since adenosine is suggested to inhibit lipolysis which could impact the respiration rate (Szillat and Bukowiecki, 1983). Furthermore, our understanding of cellular and receptor sensitivity of thirteen-lined ground squirrel brown adipocytes to adenosine could be deepened by *in vitro* testing of ADORA1 agonists and antagonists, for example CHA and PSB-36 (1-Butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione), respectively. Lastly, direct injection of adenosine, adenine nucleotides, and ADORA1 agonists and antagonists into thirteen-lined ground squirrels across their circannual cycle and at various body temperatures could illuminate any seasonal and temperature changes in adenosine sensitivity that were not observed with isolated brown adipocytes, but have been seen in other hibernating ground squirrel species (Jinka et al, 2011) as well as Syrian hamsters (Tamura et al, 2005).

CHAPTER 6: Conclusion

The thirteen-lined ground squirrel undergoes rapid shifts in body temperature during hibernation as it enters torpor for several days before returning to 12-24 hour normothermic body temperature during IBA. This results in regulated changes in the physiology of tissues that produce much of the heat required during this shift, namely BAT and skeletal muscle. To understand how the squirrel survives such extreme shifts, I focused on the impact of temperature and season on BAT and skeletal muscle. Re-warming from torpor is initiated by thermogenic BAT via non-shivering thermogenesis before skeletal muscles produce heat by shivering. As skeletal muscles shiver, energy is required in the form of ATP. The metabolism of adenine nucleotides (AMP, ADP, ATP) are a potential source of the nucleoside adenosine. Adenosine has been suggested to serve as a potential mechanism to inhibit BAT heat production by receptor-dependent processes and therefore, could be important during hypothermic torpor when additional heat is not needed.

Although there is considerable research on the role that BAT mitochondria and adipocytes play in heat generation via non-shivering thermogenesis, there are still many unknowns including the impact of temperature and season on cellular and mitochondrial function. Sampling throughout the circannual cycle, I measured respiration rates of isolated BAT mitochondria fueled by succinate at five temperatures (5, 13, 21, 29, and 37^{o}C) and brown adipocyte respiration in the presence of adenosine at two temperatures (10 and 37^{o}C). Overall, I found that the respiration rates of isolated BAT mitochondria and adipocytes are independent of season at each temperature measured *in vitro*. The response of BAT mitochondria, compared to mitochondria from a non-thermogenic

tissue, suggests there are mechanisms allowing function across a vast range of temperatures. It is possible that BAT mitochondria have some mechanism that allows function in torpor as it does in IBA across a range of temperatures that is not present in non-thermogenic tissues. However, the opposite is also possible; non-thermogenic tissues may have a mechanism that suppresses function at lower temperatures. Yet, what these mechanisms are remains to be determined. In the liver, there are seasonal changes in mitochondrial respiration rates that could be explained by changes in the activity of the ETS complexes (Brown et al, 2013; Mathers et al, 2016; McFarlane et al, 2017). Also, if any post-translational modifications to Complex II occur, they could explain the suppression of respiration rates seen (Mathers et al, 2016). Since the complexes of the ETS from BAT mitochondria do not show any differences in enzyme activity when measured at either 10 or 37^{o}C any changes in mitochondrial respiration probably occurs on the organelle has a whole instead of just the enzyme complexes (McFarlane et al, 2017). Furthermore, the mitochondrial isolation process occurs at low temperatures so any membrane remodeling enzymes would be inhibited (McFarlane et al, 2017). It would be interesting to see if any post-translational modifications occur on both BAT and liver mitochondrial complexes of the ETS and make comparisons between the two tissues.

Isolated brown adipocytes appear sensitive to adenosine regardless of season and temperature *in vitro* as demonstrated by a return of respiration rates to basal levels after significant stimulation. This could serve as a potential mechanism to inhibit heat production in BAT. However, I did not observe any seasonal changes at either

temperature. Any potential seasonal changes may have been reversed during the adipocyte isolation process since it requires extended periods of incubation at high temperatures (37^{o}C, McFarlane et al, 2017). *In vivo* studies in mice, rats, Syrian hamsters, and arctic ground squirrels suggest seasonal sensitivity to adenosine, adenine nucleotides, and adenosine agonists (Swoap et al, 2007; Zhang et al, 2006; Tupone et al, 2013; Tamura et al, 2005; Jinka et al, 2011) so it would be interesting to see if the same trend holds in thirteen-lined ground squirrels.

I also measured expression levels of four proteins from isolated skeletal muscle (AMPK, activated AMPK, AMPD1, and SLC29A1) involved in the production and transport of adenosine and the adenosine receptor on the cell surface of brown adipocytes (ADORA1) using Western blotting (Hampton et al, 2013). AMP Kinase serves as a regulator of metabolism and when activated, it aims to decrease ATP utilization and increase ATP production. AMP Kinase activation could be especially important during hibernation since activation stimulates processes such as fatty acid oxidation, mitochondrial biogenesis, and metabolic depression. AMP Deaminase 1 plays into this feedback loop by metabolizing AMP to IMP, but with low AMPD1 levels during hibernation, accumulating AMP would be available for conversion to adenosine. Adenosine can then be transported into the bloodstream via SLC29A1 and potentially bind to the adenosine receptor, ADORA1, on brown adipocytes. Once bound, this could inhibit the cascade of events that eventually results in heat production in BAT mitochondria via UCP1 thus serving as a potential mechanism to inhibit additional heat production. Two proteins, ADORA1 and AMPD1, involved in adenosine signaling and

metabolism are differentially expressed in BAT and skeletal muscle, respectively; which could impact the function of BAT and other tissues. Therefore, skeletal muscle could serve as an influential mechanism for the metabolic reduction seen in hibernation through seasonal expression levels of proteins which could influence downstream pathways involved in energy conservation and the production and regulation of heat production with adenosine. Measuring the concentration of adenosine and adenine nucleotides in skeletal muscle and the serum before and after shivering thermogenesis occurs would further our understanding on protein function in skeletal muscle.

In conclusion, these studies improve our understanding of the temperature and seasonal impact and changes in BAT and skeletal muscle, the two tissues primarily responsible for re-warming throughout hibernation in the thirteen-lined ground squirrel. Specifically, isolated BAT mitochondria and brown adipocytes appear to function independently of temperature and season when investigated *in vitro*; thus, the BAT experiments suggest both ability to function and regulation readily occur. Ability to produce heat from low ambient and body temperatures is critical throughout the hibernation season. Equally as important is the ability to control additional heat production when normothermic body temperatures are reached. Furthermore, with skeletal muscle having the potential to respond to metabolic and functional demands by serving as an endocrine-like organ makes it an ideal target for hibernators to adjust physiology through changes in one tissue. One mechanism by which those changes can be realized is through differential protein expression. The skeletal muscle studies suggest some significant seasonal changes of metabolic pathway proteins involved in mediating

intracellular adenosine levels, which is implicated in the control of heat production in BAT via ADORA1. Since hibernation involves coordinated responses from several organs, the work outlined in this thesis demonstrates the importance of not only looking at the function of each organ individually, but also how one tissue may influence the response of other tissues.

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